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## RESPONSES OF NITROGEN CYCLING TO NUTRIENT ENRICHMENT IN NEW ENGLAND SALT MARSHES OVER AN ANNUAL CYCLE

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RESPONSES OF NITROGEN CYCLING TO NUTRIENT ENRICHMENT  
IN NEW ENGLAND SALT MARSHES OVER AN ANNUAL CYCLE

BY

LEANNA RUTH HEFFNER

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE  
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2013

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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2013

## **ABSTRACT**

The excessive input of anthropogenic nutrients to coastal waters has impacted estuarine ecosystems worldwide, resulting in low oxygen conditions, increases in the severity and frequency of nuisance and harmful algal blooms, the loss of submerged aquatic vegetation, and changes to community diversity and structure. Salt marshes are ecologically important estuarine ecosystems that provide habitat for marine and terrestrial species, provide protection from storm surge, and transform nutrients at high rates. Because of these qualities there is much interest from scientific and management communities to understand the impacts of nutrient enrichment on salt marshes, as well as the potential for marshes to remove excess nutrients from estuarine systems.

Nitrogen (N) is the limiting nutrient in most coastal ecosystems and therefore studies on nutrient enrichment in marshes have largely focused on N. While decades of research have characterized the exchange of nitrogen between marshes and adjacent tidal waters, the net impact of the microbial-mediated fluxes of nitrogen gas ( $N_2$ ) is less understood. Nitrogen fixation and denitrification serve as important pathways for sources and sinks of N within the ecosystem. Nitrogen fixation is the process by which  $N_2$  gas is fixed into a biologically-available form and can be important in enhancing marsh primary production. Denitrification transforms nitrate into  $N_2$  gas, effectively removing N from the marsh. Both processes are controlled by various factors, including dissolved inorganic nitrogen (DIN) levels. Prior research has demonstrated that N fixation can be suppressed by high levels of ammonium and nitrate while denitrification is often enhanced by an increased availability of nitrate.

Many studies have examined the impact of N enrichment on denitrification and/or N fixation in salt marshes and have found varying results. While some have reported that higher DIN levels stimulated denitrification and suppressed N fixation, opposite or no relationships have also been observed. The variation in findings demonstrates that more investigation is needed, particularly because of the spatial heterogeneity of salt marshes and the methodological difficulties in measuring denitrification and N fixation. Even less is known regarding the impact of nutrient *reductions* on salt marsh biogeochemistry and N cycling. In many estuaries and coastal watersheds management actions to reduce nutrient inputs from wastewater treatment facilities and septic systems have been or will soon be implemented. Therefore it is increasingly important to better understand the response of salt marsh nutrient cycling to both nutrient enrichment and reduction.

To examine the impact of changes in N regime on salt marsh N cycling, we measured denitrification and N fixation in two marshes with varying degrees of long-term N enrichment from tidal waters. We conducted our work in Narragansett Bay, Rhode Island, which has an established down-bay gradient in estuarine nutrient concentrations. Our highly N enriched marsh was located in the Providence River Estuary, where the majority of anthropogenic N enters the Bay, and our low N marsh was located near the mouth of the Bay. To compare N cycling activity between the two marshes and to understand how activity differs seasonally, we measured denitrification and N fixation in separate sediment incubations on a monthly basis over an annual cycle (excluding winter months) from June 2011 to June 2012. Our measurements were made in intact sediment cores collected from the tidally

influenced low marsh zone dominated by short-form *Spartina alterniflora*. While this was meant to capture differences between the marshes with long-term exposure to high or low tidal N inputs, we also aimed to understand how N cycling activity would respond to changes in N regime. Therefore we additionally conducted an experiment in which sediment cores were extracted and transplanted between the marshes, along with cores that were re-planted within the same marsh (serving as experimental controls). After three months (July to October 2011) we collected the cores and in two separate incubations measured denitrification and N fixation rates.

For all of our denitrification measurements, we employed the isotope pairing technique (IPT) in which a heavy isotope nitrate ( $^{15}\text{N-NO}_3^-$ ) tracer is added to the overlying water to track the production of  $\text{N}_2$  gas. The IPT method allowed us to measure ambient denitrification, including differentiating direct denitrification from coupled nitrification-denitrification. We were also able to measure the capacity for denitrification. By measuring and distinguishing the different types of denitrification using IPT, we could comprehensively characterize the role of marsh sediments in removing tidal N (via direct denitrification) and the total capacity to denitrify when nitrate was not limiting (i.e. very high nitrate concentrations).

We found that ambient denitrification was greater at the high N marsh, due to enhanced direct denitrification stimulated by elevated levels of tidal nitrate. The difference in activity between marshes was greatest in the early fall and spring when nitrate levels seasonally peaked in the surface waters at the high N marsh. Coupled nitrification-denitrification and sediment oxygen demand were similar between sites, suggesting that sediment carbon availability was also similar. We also observed

greater denitrification capacity at the high N marsh, suggesting that the denitrifiers were better adapted to efficiently process high N inputs.

Results from the transplant experiment corroborated these findings. When sediments from the low N marsh were transplanted into the high N environment, ambient denitrification activity increased but never fully reached levels seen in sediments native to the high N marsh. Additionally, the capacity for denitrification was greatest in cores from the high N marsh that remained in their high N environment. In contrast, denitrification capacity and ambient activity decreased when cores from the high N marsh were transplanted into a low N environment. Our experiments demonstrated that N enrichment stimulated direct denitrification and suppressed N fixation, while N reductions had the opposite effects. The overall results suggest that external N inputs act as an important control on denitrification, driving short-term responses to changes in N regime, as well as shaping microbial activity on longer time scales.

For the N fixation measurements we employed the commonly used acetylene reduction assay technique, a proxy measurement that tracks the reduction of acetylene gas to ethylene by nitrogenase, the enzyme responsible for N fixation in diazotrophs. Similar to denitrification, we measured N fixation on a monthly basis over an annual cycle in intact, whole cores. We also compared incubation techniques because few salt marsh N fixation studies have employed the use of whole cores and instead have used bottle-type incubations with small sediment plug samples. In four of these monthly incubations, measurements made in whole cores were compared to concurrent sediment plug bottle incubations. Though the sediment plug incubations

yielded significantly higher rates than the whole cores, we observed greater N fixation at the low N marsh using both methods. Because carbon availability was similar between marshes, we attribute the differences between marshes to the suppression of N fixation by high tidal DIN levels at the high N marsh.

The N fixation transplant experiment also demonstrated that activity was likely suppressed at the high N marsh. Nitrogen fixation declined when sediments were transplanted from the low N marsh into an N enriched environment, but never decreased to the low levels seen in sediments native to the high N marsh. The impacts of N reduction on sediments from the high N marsh were not clear due to high variability among cores. Similar to denitrification, we observed short-term responses to changes in N regime and a potential legacy effect from long-term N availability that influenced N fixation activity.

Regarding the role of salt marshes in nitrogen removal, net  $N_2$  flux was dominated by denitrification, with direct denitrification driving differences between sites in tidal N removal. However, our observed rates of denitrification and N fixation were at the lower end of the range reported in the literature. Also, the estimated percent of N removed in tidal water per square meter of low marsh was small (5% annual average in the high N marsh and 12% in the low N marsh), owing to relatively low rates of denitrification paired with a limited amount of time that the low marsh was flooded with tidal waters. The overall trends we observed in all of our experiments, however, demonstrate that seasonal and historical N availability and changes in N regime have significant impacts on N fixation and denitrification in these marshes.



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I am also incredibly grateful to Anne Giblin and Charles Roman, who stepped up as my co-advisors following Scott's passing. You have both been wonderfully kind and supportive over the last year and a half. Thank you for doing all of the time spent doing the laborious tasks that make every advisor cringe – the revisions and edits, paperwork, conference calls, and meetings. Thank you Charley for your good

humor, for letting me always speak frankly and openly, and for going to bat for me when dealing with the “upper management”. Thank you Anne for the many years you have invested in me, even prior to Scott’s passing. Scott always said that he knew I was in good hands with you, and he was right. These last five years I have learned an incredible amount from you, thanks to your patience and your dedication and training. Salt marsh biogeochemistry is a very challenging area of study, and it is because of your mentorship that I feel proud of my accomplishments in this dissertation. Thank you SO very much.

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## **DEDICATION**

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## **PREFACE**

As described in the URI Graduate School guidelines for thesis preparation, this dissertation is organized in a manuscript format. The body of the text is divided into three sections, corresponding to the format of journal articles. All three manuscripts are co-authored by Anne Giblin, Scott Nixon, Roxanne Marino, and Charles Roman. These manuscripts will be submitted to one of the following journals: Biogeochemistry (Chapter 1); Estuarine, Coastal and Shelf, Science (Chapter 2); and Estuaries and Coasts (Chapter 3). There is one appendix at the end of this dissertation with additional details on methods and calculations.

## TABLE OF CONTENTS

ABSTRACT .....	ii
ACKNOWLEDGEMENTS .....	vii
DEDICATION .....	xiv
PREFACE .....	xv
TABLE OF CONTENTS .....	xvi
LIST OF TABLES .....	xx
LIST OF FIGURES .....	xxi

### **CHAPTER 1 - EXAMINING THE RESPONSE OF DENITRIFICATION TO NUTRIENT ENRICHMENT OVER AN ANNUAL CYCLE IN NEW ENGLAND SALT MARSHES ..... 1**

ABSTRACT .....	2
INTRODUCTION .....	4
MATERIALS AND METHODS .....	7
<i>Study Areas</i> .....	7
<i>Site Characterization</i> .....	8
<i>Isotope Pairing Technique</i> .....	11
<i>Core Collection</i> .....	13
<i>Core Incubations</i> .....	14
<i>Analytical Methods</i> .....	16
<i>Statistical Analysis</i> .....	17
RESULTS .....	18
<i>Site Characterization</i> .....	18
<i>Ambient Denitrification</i> .....	20
<i>Ambient Direct Denitrification and Coupled Nitrification-</i> <i>Denitrification</i> .....	21
<i>Denitrification Capacity</i> .....	23
<i>Nutrient Uptake and Production</i> .....	24
DISCUSSION .....	24
<i>Ambient Denitrification</i> .....	24
<i>Ambient Direct Denitrification and Coupled Nitrification-</i> <i>Denitrification</i> .....	28
<i>Denitrification Capacity</i> .....	33
<i>Nitrate Removal</i> .....	35
<i>Conclusions</i> .....	39
LITERATURE CITED .....	41
TABLES .....	46
FIGURES .....	49

**CHAPTER 2 - NITROGEN FIXATION IN NEW ENGLAND SALT  
MARSHES: EXAMINING THE IMPACT OF NUTRIENT ENRICHMENT  
OVER AN ANNUAL CYCLE AND COMPARING INCUBATION METHODS**  
..... **54**

ABSTRACT .....	55
INTRODUCTION .....	57
MATERIALS AND METHODS .....	60
<i>Study Areas</i> .....	60
<i>Acetylene Reduction Assay</i> .....	61
<i>Core Collection</i> .....	62
<i>Core Incubations</i> .....	63
<i>Whole Core Method</i> .....	63
<i>Sediment Plug Method</i> .....	67
<i>Analytical Methods</i> .....	67
<i>Statistical Analysis</i> .....	68
RESULTS .....	70
<i>Nutrients in Ambient Tidal Water</i> .....	70
<i>Sediment Oxygen Demand in Whole Cores</i> .....	71
<i>Nutrient Uptake &amp; Production in Whole Cores</i> .....	72
<i>Nitrogen Fixation in Whole Cores &amp; Sediment Plugs</i> .....	72
DISCUSSION .....	73
<i>Effect of Nutrient Enrichment on Nitrogen Fixation</i> .....	73
<i>Comparison of Incubation Methods</i> .....	77
<i>Conclusions</i> .....	83
LITERATURE CITED .....	85
TABLES .....	90
FIGURES .....	93

**CHAPTER 3 - THE IMPACT OF NUTRIENT ENRICHMENT ON SALT  
MARSH NITROGEN FIXATION AND DENITRIFICATION: A  
TRANSPLANT EXPERIMENT** ..... **99**

ABSTRACT .....	100
INTRODUCTION .....	102
MATERIALS AND METHODS .....	106
<i>Study Areas</i> .....	106
<i>Core Collection and Transplantation</i> .....	107
<i>Core Incubations</i> .....	109
<i>Nitrogen Fixation: Acetylene Reduction Assay</i> .....	109
<i>Denitrification: Isotope Pairing Technique</i> .....	110
<i>Core Incubation Set-up and Sampling Design</i> .....	111
<i>Analytical Methods</i> .....	114

<i>Statistical Analysis</i> .....	115
RESULTS .....	116
<i>Tidal Nutrient Enrichment</i> .....	116
<i>Nitrogen Fixation</i> .....	116
<i>Denitrification</i> .....	117
<i>Sediment Oxygen Demand</i> .....	118
<i>Nutrient Fluxes</i> .....	119
DISCUSSION .....	119
<i>Nitrogen Fixation</i> .....	119
<i>Denitrification</i> .....	124
<i>Net N<sub>2</sub> Flux and N Removal</i> .....	129
<i>Conclusions</i> .....	132
LITERATURE CITED .....	134
FIGURES .....	139

## **APPENDIX A – ADDITIONAL METHODS AND CALCULATION DETAILS** **..... 146**

A-1. CALCULATIONS FOR PRODUCTION/UP TAKE OF N <sub>2</sub> , ETHYLENE, AND NUTRIENTS IN INTACT CORE INCUBATIONS: CORRECTING FOR DILUTION .....	146
A-2. CALCULATIONS FOR PRODUCTION/UP TAKE OF N <sub>2</sub> , ETHYLENE, AND NUTRIENTS IN INTACT CORE INCUBATIONS: CONVERSIONS, REGRESSIONS, AND EXTRAPOLATIONS .....	149
A-3. CALCULATIONS FOR PRODUCTION OF N <sub>2</sub> IN INTACT CORE INCUBATIONS: EXAMPLE REGRESSIONS .....	151
A-4. CALCULATIONS FOR PRODUCTION OF ETHYLENE IN INTACT CORE INCUBATIONS: EXAMPLE REGRESSIONS .....	152
A-5. CALCULATIONS FOR PRODUCTION OF ETHYLENE IN SEDIMENT PLUG INCUBATIONS: CONVERSIONS AND EXTRAPOLATIONS .....	153
A-6. CALCULATION OF STANDARD ERROR OF LINEAR REGRESSIONS .....	154
A-7. BROMIDE TRACER TESTS TO MEASURE DEPTH PENETRATION OF AMENDED SEAWATER DURING PRE-INCUBATION OF INTACT SEDIMENT CORES .....	155

A-8. COMPARISON OF OVERLYING ONLY VERSUS POREWATER + OVERLYING MIX RATES FOR AMBIENT DENITRIFICATION .....	157
A-9. AMBIENT TIDAL NUTRIENTS FROM DENTIRFICATION AND NITROGEN FIXATION INCUBATIONS .....	158
A-10. SEDIMENT OXYGEN DEMAND FROM DENTIRFICATION AND NITROGEN FIXATION INCUBATIONS .....	159
A-11. TIDAL INUNDATION AND FLOODING DATA .....	160
A-12. TRANSPLANT EXPERIMENTAL DESIGN AND METHODS CONSIDERATIONS .....	162

## LIST OF TABLES

Table 1-1. Site characterization of Little Mussachuck and Fox Hill marshes .....	46
Table 1-2. Statistics results for site characterization and denitrification incubations .....	47
Table 1-3. Tidal nitrogen removal via denitrification .....	48
Table 2-1. Statistics results for nitrogen fixation incubations.....	90
Table 2-2. Depth comparison of nitrogen fixation in sediment plugs .....	91
Table 2-3. Regional literature comparisons of nitrogen fixation rates.....	92

## LIST OF FIGURES

Figure 1-1. Location of study sites for denitrification incubations.....	49
Figure 1-2. Monthly sediment oxygen demand in denitrification incubations .....	50
Figure 1-3. Monthly nitrate + nitrite concentrations in tidal water, direct denitrification, and coupled nitrification-denitrification .....	51
Figure 1-4. Monthly ambient denitrification and denitrification capacity .....	52
Figure 1-5. Monthly nitrate + nitrite and ammonium uptake and production in denitrification incubations .....	53
Figure 2-1. Location of study sites for nitrogen fixation incubations.....	93
Figure 2-2. Monthly nitrate + nitrite and ammonium concentrations in tidal water for nitrogen fixation incubations.....	94
Figure 2-3. Monthly sediment oxygen demand in nitrogen fixation incubations .....	95
Figure 2-4. Monthly nitrate + nitrite and ammonium uptake and production in nitrogen fixation incubations.....	96
Figure 2-5. Monthly nitrogen fixation measured in whole core incubations.....	97
Figure 2-3. Comparison of nitrogen fixation measured using the whole core method and sediment plug method .....	98
Figure 3-1. Location of study sites for transplant experiment .....	139
Figure 3-2. Ambient ammonium and nitrate + nitrite concentrations in tidal water for transplant experiments .....	140
Figure 3-3. Nitrogen fixation in the transplant experiment cores .....	141
Figure 3-4. Denitrification activity in the transplant experiment cores .....	142
Figure 3-5. Sediment oxygen demand in the transplant experiment cores .....	143
Figure 3-6. Nutrient fluxes in the transplant experiment cores incubated for nitrogen fixation measurements .....	144
Figure 3-7. Nutrient fluxes in the transplant experiment cores incubated for denitrification measurements .....	145

## **CHAPTER 1**

### **PREFACE**

# **EXAMINING THE RESPONSE OF DENITRIFICATION TO NUTRIENT ENRICHMENT OVER AN ANNUAL CYCLE IN NEW ENGLAND SALT MARSHES**

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**CHAPTER 1**  
**EXAMINING THE RESPONSE OF DENITRIFICATION TO NUTRIENT**  
**ENRICHMENT OVER AN ANNUAL CYCLE IN NEW ENGLAND**  
**SALT MARSHES**

**ABSTRACT**

There is much interest in understanding the potential for salt marshes to remove some of the anthropogenic nitrogen flowing into coastal systems. Nitrogen can be permanently removed via denitrification, which converts biologically available N to nitrogen gas. We measured denitrification using the isotope pairing technique (IPT) in sediment cores taken from two marshes located at the extremes of the nutrient concentration gradient in Narragansett Bay, Rhode Island. Ambient denitrification rates and total denitrification capacity were measured on a monthly basis during the spring, summer, and fall for one year. By using IPT, we distinguished between rates of direct denitrification of nitrate in the tidal water versus coupled nitrification-denitrification of nitrate produced within the sediment. We found that total capacity and ambient denitrification rates were significantly greater at the highly N enriched marsh. The maximum differences between the marshes generally occurred during early fall and spring, coinciding with the greatest water column nitrate values. In addition, seasonal nitrate availability in the tidal water corresponded with seasonal changes in direct denitrification. In contrast, both marshes exhibited similar rates of

coupled nitrification-denitrification and sediment oxygen uptake. Total denitrification capacity was higher than ambient denitrification, often by an order of magnitude, suggesting that denitrifiers at both sites are nitrate-limited. In addition, long-term exposure to high nitrate concentrations resulted in higher denitrification capacity. We conclude that anthropogenic N enrichment affects marsh denitrification rates on short and long-term time scales, stimulating instantaneous rates of direct denitrification on a seasonal basis and, following prolonged exposure to high nitrate concentrations, increasing the overall capacity for denitrification.

## INTRODUCTION

In the last several decades, human activities have increasingly impacted coastal ecosystems. High anthropogenic nutrient loading resulting from sewage outflows and agricultural and urban runoff to near-shore waters has received significant attention from coastal scientists and managers in the last quarter-century (National Research Council 2000; (Howarth et al. 2002). Coastal eutrophication resulting from increased anthropogenic nutrient enrichment has negatively impacted estuarine ecosystems worldwide, including chronic and severe hypoxia and anoxia, loss of submerged aquatic vegetation, changes in benthic communities, and an increase in the frequency and severity of algal blooms (Nixon 1995; Vidal et al. 1999; Rabalais and Nixon 2002). Salt marshes are ecologically important coastal ecosystems, with high rates of biogeochemical cycling of nutrients, and that serve as a link between freshwater, terrestrial, and marine communities. These ecosystems provide important nursery habitat, foraging grounds, and refuge from predators (Teal 1962; Valiela and Teal 1979; Boesch et al. 1984; Deegan and Garritt 1997; Craig and Crowder 2000). There is much interest in understanding how salt marsh ecosystems are affected by high nutrient loading from adjacent terrestrial sources and tidal flooding from eutrophic estuarine waters (Gedan et al. 2009).

The potential for salt marshes to intercept and remove anthropogenic nitrogen (N) via gaseous losses and burial is especially important to understand, as marshes may act as buffers to the eutrophication of nearby coastal waters (Teal and Howes 2000; Valiela and Cole 2002; Fisher and Acreman 2004). Denitrification, the

microbial-mediated process by which nitrate is converted to  $N_2$  gas, is typically high in salt marsh sediments compared to other marine sediments and is an important mechanism for N removal (Hopkinson and Giblin 2008). Because denitrification in salt marshes is typically limited by nitrate availability, recent studies have examined the impact of N enrichment on denitrification rates and capacity, with widely varying results. For example, some studies have found that experimental fertilization and high nutrient loading in marshes resulted in higher denitrification (Lee et al. 1997; Teal and Howes 2000; Hamersley and Howes 2005; Aelion and Engle 2010). Negative relationships between N enrichment and denitrification, insignificant trends, or spatially dependent responses to nutrient inputs have also been observed in several other studies (Nowicki et al. 1999; Davis et al. 2004; Wigand et al. 2004; Tuerk and Aelion 2005; Caffrey et al. 2007; Koop-Jakobsen and Giblin 2010).

These inconsistencies could be due to the high variability of denitrification within and among marshes or may result from methodological differences among studies. There are various ways to measure denitrification, each with their own advantages and disadvantages (Groffman et al. 2006). Many methods do not directly measure ambient denitrification. Instead they provide an indicator of denitrification activity such as denitrification potentials (capacity under ideal conditions), net  $N_2$  flux (which accounts for  $N_2$  inputs as well as losses), denitrification enzyme activity (DEA), and the abundance of the microbial genes responsible for denitrification. While these approaches provide important insights, they may not adequately capture the specific denitrification activity associated with variability in N inputs. Even directly measuring total ambient denitrification may not provide a comprehensive

assessment. Denitrification in marshes reduces nitrate that originates from two sources: nitrate produced within the sediment (coupled nitrification-denitrification) or external, predominately anthropogenic nitrate (direct denitrification) that flows into the marsh via groundwater and tidal creeks (Howes et al. 1996). Measuring direct and coupled denitrification separately provides a more comprehensive way to examine the impact of external nitrate enrichment on marshes, though only a few studies have used this approach (e.g. (Nowicki et al. 1999; Hamersley and Howes 2003; Koop-Jakobsen and Giblin 2010). Furthermore, while many studies have examined the spatial variability of N loading impacts on denitrification within salt marshes (e.g. comparing high marsh, low marsh, creek banks, bare patches, surface sediment, rhizosphere; e.g. (Kaplan et al. 1979; Aziz and Nedwell 1986; Koch et al. 1992; Eriksson et al. 2003; Koop-Jakobsen and Giblin 2010), the seasonal variation in denitrification under various N regimes is less well understood (Hopkinson and Giblin 2008).

The goal of this study was to examine the impact of tidal N concentrations on marsh denitrification rates, and how this varies seasonally, by comparing two marshes at the extremes of a nutrient gradient in Narragansett Bay, Rhode Island over an annual cycle. We quantified direct and coupled nitrification-denitrification separately to determine if any differences we observed were driven by the reduction of nitrate in tidal waters versus coupled nitrification-denitrification. In addition to measuring the response of ambient denitrification to differences in N enrichment, we also studied denitrification capacity under conditions when nitrate was not limiting.

Denitrification in intact sediment cores collected from the tidally-influenced short-form *Spartina alterniflora* zone, as well as sediment oxygen demand, and

nutrient (ammonium and nitrate + nitrite) fluxes were measured monthly in the summer, fall, and spring. We amended the cores with a  $^{15}\text{N}$  isotopic nitrate tracer and used the isotope pairing technique (Nielsen 1992) to calculate total denitrification capacity and ambient (direct vs. coupled) denitrification rates. To determine possible factors influencing trends in denitrification between sites, we measured sediment carbon and nitrogen content, aboveground plant biomass, ambient tidal nitrate concentrations, and tidal flooding of the marsh platform.

## **MATERIALS AND METHODS**

### ***Study Areas***

Narragansett Bay is a small (328 km<sup>2</sup>) temperate estuary that lies along a north-south orientation to Rhode Island's coastline (Nixon et al. 1995). The majority of anthropogenic nutrients enter the Bay in the urbanized upper reaches via riverine inputs and wastewater treatment plant effluent, resulting in a north-south gradient in nutrient concentrations (Nixon et al. 1995; Oviatt et al. 2002; Oviatt 2008). At the head of the Bay, the Providence River Estuary is highly eutrophic, often experiencing seasonal summer hypoxia. In contrast, the mid and lower Bay have much lower nutrient and surface chlorophyll-*a* concentrations (~70-75% less; (Oviatt et al. 2002).

We chose two marshes as study sites that lie at the ends of the north-south nutrient gradient (Fig. 1-1). Little Mussachuck marsh (Barrington, RI; 4.4 hectares) is located on the eastern shore of the Providence River Estuary and is flooded with

nutrient enriched, brackish (~19-29 psu) waters (Table 1-1). The landward border of Little Mussachuck is lined with a small patch of forest and a few residences connected to sewers. The marsh, which lies 0.5 km from a golf course and adjacent to a residential neighborhood containing well-fertilized lawns (*pers. comm.* with homeowners), very likely receives additional nitrogen-rich inputs from surface runoff and groundwater. Fox Hill marsh (Jamestown, RI; 10.0 hectares) is located 26 km downstream in the lower Bay and is flooded with low nutrient, more saline (~31 psu) water (Table 1-1). It is surrounded by relatively undeveloped land that includes a small pasture farm, as well as a beach and a town park reserved for seasonal recreational camper vehicles.

Both sites exhibit typical salt marsh zonation, with tall-form *Spartina alterniflora* bordering the creek bank edges, a larger expanse of short-form *S. alterniflora* in the low marsh, and *Spartina patens* dominating the high marsh. For this study we chose to sample the regularly flooded short-form *S. alterniflora*, because it receives more tidal N enrichment than the less frequently flooded *S. patens* zone in the high marsh. We did not sample along the creek banks among tall-form *S. alterniflora* due to the highly variable and patchy sediment structure and the minimal area coverage of this vegetated zone.

### ***Site Characterization***

Because the goal of this study was to understand the effects of nitrogen enrichment on salt marsh denitrification activity, we measured additional characteristics of our study sites to compare and contrast the marshes. This provided

us with an understanding of factors influencing denitrification activity, and their role in producing potential site differences. We measured sediment oxygen demand, carbon and nitrogen content in the sediments, aboveground biomass of the grasses, surface tidal water nitrate + nitrite and ammonium concentrations, and tidal inundation of the short *S. alterniflora* zone at each marsh.

To compare microbial C availability between sites, we measured sediment oxygen demand (SOD) in the same cores used to measure denitrification, as well as additional concentrations from cores collected monthly over the same annual cycle for a separate study to measure nitrogen fixation (Ch. 2). In total, sediment oxygen demand was measured in 19 bi-monthly incubations as part of the site characterization. Oxygen concentrations were measured 3-4 times throughout each incubation using a Hach HQ30 LDO probe inserted through a small, stoppered opening in the lids of the cores (Hach Company). Although over half of microbial sediment metabolism occurs as sulfate reduction in salt marshes, most of the sulfide produced is eventually oxidized, and therefore measuring SOD can capture this anaerobic metabolism (Howarth and Hobbie 1982). However the majority of sulfides are oxidized during the growing season, which produces a temporal offset of oxygen uptake and total system respiration (Giblin and Howarth 1984). We argue that using SOD to compare C availability between sites in our study is robust because 1) we measured SOD throughout the majority of the year (including most of the growing season) and 2) our measurements of sediment activity (denitrification, nutrient uptake, etc.) were concentrated near the sediment surface. It should be noted that we are likely not accounting for total deep sediment metabolism.



Additionally, we measured sediment carbon and nitrogen content and C:N ratios in 5cm diameter cores collected from each site in August 2009 and August 2010. These cores and those extracted later for denitrification rates were taken from the same general area of each marsh. Following collection, cores were cut at 3 cm intervals to a depth of 15 cm and dried at 60°C until constant weight. The sediment from each 3 cm depth-interval was ground with a Wiley mill. An elemental instrumental analyzer (Thermo Scientific Flash 2000) was used to measure C and N content.

We measured the aboveground end-of-season biomass of the marsh grasses in September 2010. We randomly sampled six quadrats (25 cm x 25 cm) within the low marsh zone. The areas sampled were comprised of stands of *S. alterniflora* at Little Mussachuck, and at Fox Hill some quadrats contained a mixture of *S. patens* and *S. alterniflora*. Within the quadrats we collected all aboveground plant material by cutting the stems at their base, separated the material into live, dead, and litter, and dried it at 60°C until constant weight. Biomass was determined by measuring the dry weight of all aboveground live plant material within a standardized area.

We collected samples of surface seawater located adjacent to the marshes to measure tidal nutrient concentrations on a bi-monthly basis. To measure the tidal inundation in the sampling area of each marsh we placed a HOBO U20 Water Level Logger on the sediment surface (Onset Computer Corporation). The water depth above the marsh surface was recorded every 15 minutes over the course of two lunar cycles from late August through late October 2012. Data derived included depth and duration of marsh surface flooding. Water level data were corrected for atmospheric

pressure recorded by a separate HOBO logger placed at the nearby Graduate School of Oceanography at the University of Rhode Island (Narragansett, RI).

### *Isotope Pairing Technique*

To measure denitrification we used the isotope pairing technique (IPT), which uses an isotope nitrate tracer ( $^{15}\text{N-NO}_3^-$ ) to track the production of  $\text{N}_2$  gas (Nielsen 1992). Because it is extremely difficult to directly measure the ambient production of  $^{28}\text{N}_2$  gas ( $^{14}\text{NO}_3^- + ^{14}\text{NO}_3^-$ ), the production of  $^{29}\text{N}_2$  ( $^{14}\text{NO}_3^- + ^{15}\text{NO}_3^-$ ) and  $^{30}\text{N}_2$  ( $^{15}\text{NO}_3^- + ^{15}\text{NO}_3^-$ ) are measured instead. Using the IPT equations (listed below), we can use the  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  production rates to back-calculate the original production of ambient  $^{28}\text{N}_2$  gas. In addition to measuring ambient denitrification ( $D_{14}$ ) the IPT method also has the added advantage of being able to break down ambient denitrification activity into direct denitrification ( $D_w$ ) and coupled nitrification-denitrification ( $D_n$ ). This allowed us to better understand the role of ambient denitrification in removing external nitrate from the water-column (via direct denitrification) compared to the reduction of nitrate internally produced within the system (via coupled nitrification-denitrification). Furthermore, by adding high concentrations of the  $^{15}\text{N-NO}_3^-$  tracer to the overlying water, we were able to measure the capacity for denitrification under conditions of unlimited nitrate (total denitrification:  $D_{Total}$ ).

IPT Equations (Steingruber et al. 2001):

(1)

$$D_{15} = r_{29} + 2 * r_{30}$$

(2)

$$D_{14} = D_{15} * \frac{r_{29}}{2 * r_{30}}$$

(3)

$$D_{Total} = D_{15} + D_{14}$$

(4)

$$D_w = D_{15} * \frac{{}^{14}\text{NO}_3^-}{{}^{15}\text{NO}_3^-}$$

(5)

$$D_n = D_{14} - D_w$$

Where:

$D_{15}$  = Denitrification of  ${}^{15}\text{N-NO}_3^-$  tracer

$D_{14}$  = Denitrification of  ${}^{14}\text{N-NO}_3^-$  (total ambient denitrification)

$D_{Total}$  = Total denitrification capacity

$D_w$  = Ambient direct denitrification

$D_n$  = Ambient coupled nitrification-denitrification

$r_{29}$  = Production rate of  ${}^{29}\text{N}_2$

$r_{30}$  = Production rate of  ${}^{30}\text{N}_2$

The IPT method has some underlying important assumptions that must be considered: 1) shortly following the addition of the  ${}^{15}\text{N-NO}_3^-$  tracer, a stable nitrate concentration gradient is established across the sediment-water interface, 2) ambient denitrification of naturally-occurring nitrate is not affected by the addition of the tracer, and 3) the  ${}^{15}\text{N-NO}_3^-$  tracer uniformly mixes with naturally-occurring  ${}^{14}\text{N-NO}_3^-$ . We also assumed that nearly all of the measured  $\text{N}_2$  production would originate from denitrification. Although anammox also produces  $\text{N}_2$  (directly by reducing ammonium) we expected that anammox activity would be minimal in our cores based on previous findings in other salt marshes and coastal sediments that have reported

extremely low rates of anammox (Engström et al. 2005; Koop-Jakobsen and Giblin 2009).

### ***Core Collection***

We measured denitrification activity in intact sediment cores collected from each marsh from June 2011 to June 2012. Sediment cores were collected within a 0.5 hectare area of each marsh at monthly intervals over an annual cycle (excluding winter months of January, February and March when the marshes are often frozen). Every month 4-5 cores (10 cm inner diameter, 15 cm deep) were carefully extracted from each marsh at low tide in the short *S. alterniflora* zone. In September, due to logistical constraints, we collected four cores from each site. We collected cores in between plant shoots in order to exclude aboveground plant biomass in the cores, as the gases produced by the leaves could have an effect on the ratio of dissolved gases in the overlying water during incubations. Although the cores were bare on the surface, the sediments contained many roots and rhizomes and sometimes very small, budding shoots. Therefore we considered the sediments to be “vegetated”. To extract the marsh sediment, core tubes with a sharpened bottom-edge were hammered down to depth, dug out carefully with the sediment intact, and stored in coolers. We then transported the cores to a temperature-controlled environmental chamber at the Graduate School of Oceanography where they were left to drain (mimicking low tide) overnight at ambient soil surface temperatures. Following core extraction from the marsh we collected tidal water at the marsh creek inlets to use as overlying water during the incubations, which was subsequently filtered to remove particles greater

than 0.2 microns. We also used the collected water to determine ambient surface water nutrient concentrations at each site.

### *Core Incubations*

The day after collection, the sediment cores were incubated under flooded conditions in the dark at ambient soil temperatures. Tidal seawater collected from each site was amended with 15-potassium nitrate (~99.9%  $^{15}\text{N}$ ) tracer (160 $\mu\text{M}$  of added  $^{15}\text{N-NO}_3^-$ ). Initially, sediments were “pre-incubated” for 2-3 hours by allowing tracer-amended overlying seawater to flood the pore spaces of the sediment while allowing some water to slowly drain out of the bottom of the cores. We expected an initial “lag-period” in  $\text{N}_2$  gas production would initially occur due to the time required for the amended water to diffuse and drain into the sediments. Therefore the pre-incubation was done in order to allow  $^{15}\text{N-NO}_3^-$  tracer to saturate the sediments prior to taking our initial measurements of  $\text{N}_2$  gas production and avoid capturing this initial “lag-period”. Preliminary tests using a bromide tracer in the overlying water showed that pre-incubation periods of 2 hours allowed for the tracer-amended seawater to penetrate the sediments to a depth of 8 cm (Appendix A-7).

Following the pre-incubation, the cores were filled (no headspace) with ~3L of 160 $\mu\text{M}$   $^{15}\text{N-NO}_3^-$  tracer-amended seawater collected from the respective marshes, resulting in a water-column 43 cm deep. The cores were then capped with gas-tight lids fitted with sampling ports and incubated for a period ranging from 10-25 hours. The incubation times varied due to temperature effects on oxygen uptake rates, as we did not allow dissolved oxygen concentrations in the overlying water to drop below 4

mg/L. Floating magnetic stir bars placed in the middle of the cores ensured mixing of the water column during the incubation period. We collected water samples throughout the incubation to analyze for  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  production and nutrient concentrations (ambient  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$ , as well as the  $^{15}\text{N}\text{-NO}_3^-$  tracer). Overlying water samples were collected using a gravity flow-through system in order to replace any water sampled.

During short incubations as employed in this study the porewater does not come to equilibrium with the overlying water so measuring  $\text{N}_2$  production in the overlying water samples alone does not fully capture denitrification activity occurring subsurface. Therefore, after taking samples from the overlying water we collected porewater from the top 3-6 cm of each core to analyze for concentrations of  $\text{N}_2$  gas and  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$ ). To collect porewater, we destructively sampled (and sacrificed) one core at each time point from both marshes throughout the incubation. This was done by gently breaking up the top layer of sediment with a large metal fork and mixing the sediment and porewater into the overlying water. This porewater-overlying water mixture was sampled for  $\text{N}_2$  production and nutrient concentrations. Because this method captured more activity within the sediments (Appendix A-8), we only reported the data from the porewater-overlying mix and did not include data from the overlying water. To calculate the volume of porewater in this mixture, a known amount (3-5 mL) of concentrated bromide tracer was added to the water column immediately prior to disturbing the sediment (Appendix A-1). Small samples (6 mL) for bromide analysis were taken before and after destructive porewater sampling and stored at  $4^\circ\text{C}$  until analysis. Though this method of breaking up the sediment did not

capture denitrification occurring deep in the rhizosphere, a previous study in another New England salt marsh has shown that denitrification rates are mainly impacted by nitrate in the tidal water in the top 5 cm of vegetated marsh sediments (Koop-Jakobsen and Giblin 2010).

Samples for analysis of  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  production (12 mL) were stored in gas-tight Exetainers (Labco Limited), fixed with 50  $\mu\text{L}$  of zinc chloride, and stored underwater. Nutrient samples (60 mL) were filtered through 0.45  $\mu\text{M}$  Whatman glass microfiber filters, stored in acid-washed polyethylene bottles, and frozen at  $-15^\circ\text{C}$  until analysis. We also measured oxygen concentrations 3 – 4 times throughout the incubations using a Hach HQ30 LDO probe inserted through a small, stoppered opening in the lids of the cores. We ended the incubations before the oxygen levels dropped to near-hypoxia (below 4 mg/L). Water temperatures of the cores were also recorded at each sampling event using the Hach probe.

### *Analytical Methods*

Dissolved gas concentrations were measured on a quadrupole mass spectrometer without gas equilibration using a membrane inlet system (Kana et al. 1994). Concentrations of ammonium and nitrate in ambient tidal water, as well as the overlying water and porewater collected during incubations were quantified using a Lachat Instruments Quik Chem 8000 flow injection analyzer. Bromide concentrations used to track the volume of overlying versus porewater in our mixed samples were analyzed using an 861 Advanced Compact Ion Chromatograph with a Metrosep A supp 5 column.

### *Statistical Analysis*

To estimate  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  production, we sacrificed one sediment core per site during each sampling event throughout the incubation. Rates of  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  production were determined for each site by calculating the slope of linear regressions (4-5 point minimum) of concentrations plotted over incubation time (see Appendix A-3 for sample regressions). We did not need to estimate the contribution of  $\text{N}_2$  produced during the “pre-incubation” because the slope of the regression only included measurements from the sacrificed cores, with  $\text{N}_2$  from the first sampling event serving as “time zero” measurements. Similar to  $\text{N}_2$  production, we also calculated the rates of nutrient production and uptake and sediment oxygen demand using linear regressions. Total ambient denitrification ( $D_{14}$ ), direct ( $D_w$ ) and coupled ( $D_n$ ) denitrification, and denitrification capacity ( $D_{Total}$ ) were then calculated using the IPT equations previously outlined. Denitrification rates, nutrient production and uptake, and sediment oxygen demand were then corrected for dilution (which resulted from the gravity flow-through sampling of overlying water) and standardized by water volume and sediment area (see Appendices A-1 and A-2 for calculations).

The ten months of measured rates were used to test for differences between sites and among seasons. To test for seasonal trends, we grouped monthly rates together into three distinct seasons: Summer (July, August, September); Fall (October, November, December); Spring (April, May, June). Our determination of the seasons was based on general seasonal cycles in surface water nitrate previously observed in Narragansett Bay (Krumholz 2012). We tested for significant site and seasonal differences in denitrification rates, sediment oxygen demand, ambient nitrate



concentrations, and nutrient uptake and production using two-way ANOVAs or Friedman non-parametric tests for non-normally distributed data (Table 1-2). One-way ANOVAs were generally used to test for differences between marshes in site characterization data. When appropriate, some data sets were square root or log transformed to obtain normality. In cases with data that were distributed normally but had unequal variances, we used Welch ANOVA's. Relationships between variables were tested using multiple regressions. Standard errors of the linear regressions (i.e. production and uptake rates) were generated using regression analyses on Microsoft Excel (see Appendix A-6 for equations). We ran all other tests using JMP Statistical Software (v. 10.0) and SAS Statistical Software (SAS Institute, Inc.).

## **RESULTS**

### ***Site Characterization***

We compared various characteristics of the two study sites, Little Mussachuck (LMK) marsh and Fox Hill (FOX) marsh, to better understand site-specific controls on trends in denitrification activity. Sediment oxygen demand (SOD) measured bi-monthly for site characterization was statistically similar between our two study sites but varied significantly over time (Tables 1-1 and 1-2). Oxygen uptake ranged from 24.7 to 94.3 mmol m<sup>-2</sup> d<sup>-1</sup>, and the annual averages were similar between sites (53.3 ± 3.6 mmol m<sup>-2</sup> d<sup>-1</sup> at Little Mussachuck and 46.9 ± 2.9 mmol m<sup>-2</sup> d<sup>-1</sup> at Fox Hill). In the incubations for measuring denitrification, SOD rates varied positively with incubation temperature (8-23°C) over time (regression analysis,  $r^2 = 0.22$ ,  $p = 0.04$ ), with the

exception of late summer (August and September) when temperatures remained high yet SOD dropped to minimum rates (Fig. 1-2). The lowest oxygen demand occurred during coldest months in the fall. We observed differences in sediment carbon and nitrogen content, with higher percent carbon and nitrogen measured in the top 15cm of sediment at Little Mussachuck than Fox Hill (Table 1-2). Carbon to nitrogen ratios (C:N), however, were statistically similar between sites.

The two sites received significantly different concentrations of nutrients in flooding tidal waters, with 2-4x higher annual mean concentrations (averaged over the 10 month period of this study) of nitrate + nitrite and total dissolved inorganic nitrogen at Little Mussachuck compared to Fox Hill (Tables 1-1 and 1-2). Seasonal patterns in nitrate concentrations were significant, with low levels during the summer and spring, and maximum concentrations occurring in the fall (Fig. 1-3A). At Fox Hill, however, nitrate concentrations remained at near-zero levels in summer and spring, whereas nitrate at Little Mussachuck generally remained around 5 $\mu$ M. Even more striking was the difference between tidal nitrate concentrations in the fall, with a maximum of 26.2 $\mu$ M at Little Mussachuck, nearly 3x higher than the maximum of 9.4 $\mu$ M at Fox Hill. Other studies documenting annual cycles of surface nutrients in various regions of Narragansett Bay show very similar seasonal trends and concentrations of nitrate in waters near Fox Hill and Little Mussachuck marshes (Oviatt 2008; Krumholz 2012). Due to the higher concentrations of nitrate year-round at Little Mussachuck, we assumed that tidal N inputs to this marsh are much higher compared to those at Fox Hill. Although Little Mussachuck had higher tidal N concentrations, and presumably higher N inputs, than Fox Hill, aboveground end-of-

season biomass in the low marsh was statistically similar at both sites ( $407 \pm 5 \text{ g m}^{-2}$  at Little Mussachuck;  $475 \pm 40 \text{ g m}^{-2}$  at Fox Hill; Tables 1-1 and 1-2).

The general duration and depth of tidal flooding over the two month lunar cycle were similar, with high tides flooding the low marsh zone during spring tides, and minimal or no flooding during neap tides, which resulted in fewer flooding events per lunar cycle (see Appendix A-11 for tidal flooding trends over time). Also, during each high tide flooding event both sites were inundated on average for approximately 3 hours (Table 1-1). Fox Hill flooded more frequently, however, and had a significantly greater mean high water depth than Little Mussachuck (Table 1-2). Although the low marsh was flooded for less time each month at Little Mussachuck (15% less than Fox Hill), the N enrichment in Little Mussachuck from tidal waters would undoubtedly have been much higher than Fox Hill, as evidenced by the greater magnitude of differences in DIN concentrations (2-4x higher at Little Mussachuck than Fox Hill).

### ***Ambient Denitrification***

Overall ambient denitrification ( $D_{14}$ ) was greater at the high N marsh, Little Mussachuck, largely due to high rates in the spring and fall (Fig. 1-4A). Whereas activity peaked with high rates (above  $0.7 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) at Little Mussachuck in the mid fall (October and November) and late spring (May and June 2012), ambient rates remained relatively low year-round at Fox Hill, the low N marsh. Both sites exhibited similar rates when Little Mussachuck activity dropped to  $0.1 - 0.46 \text{ mmol m}^{-2} \text{ d}^{-1}$ , occurring in summer (June to August) when surface water nitrate levels were low, and

during the cold months in late fall and early spring (December and April). Due to the seasonal swings in ambient denitrification at Little Mussachuck, the range of rates was much greater ( $0.09 - 1.03 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) compared to the range at Fox Hill ( $0.17 - 0.53 \text{ mmol m}^{-2} \text{ d}^{-1}$ ), though seasonal variation was not statistically significant (Table 1-2). The difference in annual ambient denitrification between sites was significant, however, with statistically greater activity at Little Mussachuck than Fox Hill, with average rates of  $0.58 \pm 0.09 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Little Mussachuck and  $0.35 \pm 0.03 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Fox Hill. We found significant positive relationships with ambient denitrification and ambient tidal  $\text{NO}_{3+2}^-$ ,  $\text{NH}_4^+$ , and DIN (multiple regression analysis,  $p = 0.05$ ,  $0.01$ , and  $0.02$ , respectively), though the relationships were weak ( $r^2 = 0.20$ ,  $0.32$ , and  $0.27$ , respectively).

#### ***Ambient Direct Denitrification and Coupled Nitrification-Denitrification***

Using the IPT equations, we separately calculated direct denitrification ( $D_w$ ) of the ambient nitrate in the water-column and the coupled nitrification-denitrification ( $D_n$ ) of ambient nitrate produced in the sediment porewater (Fig. 1-3B, C). At Fox Hill, the large majority of ambient denitrification ( $D_{14}$ ) occurred as coupled nitrification-denitrification, averaging 95% of ambient activity over the annual cycle. Direct denitrification was negligible at Fox Hill, with the exception of the fall season (October to December) when it comprised 14-17% of total ambient denitrification. At Little Mussachuck, however, direct denitrification contributed significantly to ambient rates, comprising up to 59% of total ambient activity during the fall, and averaging 25% of ambient denitrification over the annual cycle. A comparison of the sites

showed that direct denitrification was significantly higher at Little Mussachuck than Fox Hill, but coupled rates were similar between the two marshes (Table 1-2 and Fig. 1-3B, C).

The seasonal variability of denitrification of water-column nitrate was significant over time (Friedman test,  $F_{2, 16} = 9.86$ ,  $p = 0.002$ ), and coupled with ambient tidal nitrate and water temperature at both sites (multiple regression,  $r^2 = 0.73$ ,  $p < 0.0001$ ; Fig. 1-3A, B). Ambient nitrate concentrations and direct denitrification concurrently peaked in the fall, and remained fairly low throughout the summer and spring. With the exception of the fall, direct denitrification at Fox Hill was at or near zero activity. However, even during the fall, direct denitrification at Fox Hill remained fairly low in comparison to Little Mussachuck (Fig 1-3B). For example, the peak rate of  $0.05 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Fox Hill was an order of magnitude lower than the maximum of  $0.60 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Little Mussachuck. Over the course of the 10 months measured annual mean direct denitrification at Little Mussachuck was 10x greater than Fox Hill ( $0.16 \pm 0.06$  and  $0.015 \pm 0.007 \text{ mmol m}^{-2} \text{ d}^{-1}$ , respectively).

In contrast to direct denitrification, coupled nitrification-denitrification ( $D_n$ ) was similar between the two marshes throughout most of the year, with moderate rates year-round. Though we observed a peak in September and a second peak at Little Mussachuck in May and June 2012, seasonal differences were not significant (Table 1-2 and Fig. 1-3C). Coupled denitrification averaged over the annual cycle averaged  $0.41 \pm 0.07 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Little Mussachuck and  $0.33 \pm 0.03 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Fox Hill. Higher rates of coupled denitrification at Fox Hill, peaking at  $0.53 \text{ mmol m}^{-2} \text{ d}^{-1}$  in September, starkly contrasted with very low rates of direct denitrification, whereas

the two pathways for ambient denitrification at Little Mussachuck contributed fairly evenly.

### ***Denitrification Capacity***

Denitrification capacity ( $D_{Total}$ ), which accounts for the denitrification of the added  $^{15}\text{N-NO}_3^-$  tracer in addition to ambient  $^{14}\text{N-NO}_3^-$  in the overlying and porewater at each site, represents a measure of denitrification under conditions when nitrate is not limiting but with all other conditions unchanged. Total denitrification capacity was considerably higher than ambient denitrification rates at both marshes, often by an order of magnitude (Fig. 1-4). In contrast to ambient rates, denitrification capacity at both marshes remained high throughout the summer. Seasonal variation was significant at both marshes, though more dramatic at Little Mussachuck, as evidenced by the larger range in rates ( $1.63 - 6.07 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Little Mussachuck,  $1.03 - 3.60 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Fox Hill; Table 1-2). Additionally, denitrification capacity was significantly related to temperature (regression analysis,  $p = 0.03$ ), although the relationship was weak ( $r^2 = 0.24$ ). Denitrification capacity generally differed between the two marshes when Little Mussachuck rates were highest (July-Oct and May). Similar to ambient rates, denitrification capacity over the annual cycle was significantly greater at Little Mussachuck, with average rates of  $4.38 \pm 0.52 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Little Mussachuck and  $2.46 \pm 0.27 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Fox Hill.

### ***Nutrient Uptake and Production***

The net uptake and production of nitrate and ammonium in the cores were similar between sites (Table 1-2 and Fig. 1-5). However, during the fall when direct denitrification peaked at Little Mussachuck, nitrate-nitrite consumption (which included uptake of ambient nitrate-nitrate and the  $^{15}\text{N}$ -nitrate tracer) was at a maximum and seasonally averaged uptake in the fall significantly differed between marshes. Nitrate uptake did not vary seasonally throughout the annual cycle, though rates ranged greatly from  $-2.1$  to  $-33.0 \text{ mmol m}^{-2} \text{ d}^{-1}$  (negative numbers indicate uptake, whereas positive values would have represented nitrate production). Nitrate consumption was especially high in the spring, particularly in June 2012 when SOD was also high. Over the annual cycle, the cores showed net uptake and production of ammonium, and seasonal variation was not significant. Interestingly, ammonium fluxes at Little Mussachuck positively varied with coupled nitrification-denitrification (regression analysis,  $r^2 = 0.66$ ,  $p < 0.0001$ ), with the highest rates of coupled activity corresponding with the greatest amounts of ammonium fluxing from the sediment to the water column.

## **DISCUSSION**

### ***Ambient Denitrification***

It is well established that denitrification rates in marine sediments and salt marshes are primarily controlled by nitrate, oxygen and carbon availability

(Wallenstein et al. 2006; Hopkins and Giblin 2008). A review by Wallenstein et al. (2006) examined the environmental controls on denitrifying communities and found that nitrate acts as the main limiting factor in marine systems, affecting denitrification rates instantaneously. Our results indicate that tidal nitrate concentrations in Narragansett Bay marshes play an important role in overall denitrification. In our comparison of two marshes exposed to varying degrees of N enrichment, we observed significantly greater rates of ambient denitrification (ambient direct plus coupled;  $D_{14}$ ) at the high N marsh (Little Mussachuck), with 65% greater annual mean denitrification than the low N marsh (Fox Hill; Fig. 1-4A). Over the course of an annual cycle, Fox Hill rates remained relatively low and constant year-round. Denitrification in the summer was relatively low at Little Mussachuck, and rates were similar between the two marshes at that time. The differences in ambient denitrification rates were seasonal, occurring in the fall and spring during periods of peak activity at Little Mussachuck.

Other studies in salt marshes have also found similar impacts of N enrichment on denitrification activity. Using the IPT method Koop-Jakobsen and Giblin (2010) found that ambient denitrification was much higher in the *Spartina patens* zone of the marsh platform and tidal creeks of a highly fertilized marsh compared to a nearby reference marsh. Howes et al. (1996) also reported that increased N fertilization stimulated net denitrification ( $N_2$  flux), and later Hamersley and Howes (2005) found high rates of coupled nitrification-denitrification using  $^{15}N-NH_4^+$  tracers in the same marsh. In contrast, Tuerk and Aelion (2005) measured potential denitrification measured using the acetylene block technique and reported no difference between high



and low N enriched marshes. In Narragansett Bay marshes in particular, previous studies have observed trends that either contrast or agree with our findings. Davis et al. (2004) found an inverse relationship between modeled watershed N loading and summertime net N<sub>2</sub> fluxes (which includes N-fixation) in bare and vegetated marsh sediments. In addition, a marsh fertilization study in Narragansett Bay conducted during the summer found no significant differences between fertilized and control plots in net N<sub>2</sub> flux, although potential denitrification was higher in fertilized sediments (Wigand et al. 2004; Caffrey et al. 2007). A third study conducted in the spring and fall in Narragansett Bay marshes observed greater denitrification enzyme activity (DEA) associated with high N loading in sediments within the high marsh (dominated by *Spartina patens*), though no relationship to N loading was found in low marsh sediments (Wigand et al. 2004). The contrasting results between studies may be due to differences in seasonal timing, spatial sampling, and methods employed to measure denitrification.

In general, nitrate availability tends to control seasonal variability of denitrification, although low temperatures can also be a limiting factor (Kaplan et al. 1977; Kaplan et al. 1979; Koch et al. 1992; Eriksson et al. 2003). The seasonal trends we observed suggest that ambient denitrification at Little Mussachuck were primarily controlled by tidal nitrate levels during warmer months and by temperature during colder months. The seasonal variation in sediment oxygen demand (SOD) at both marshes was largely tied to temperature changes (with the exception of August and September), indicating that microbial activity was limited during colder months (Fig. 1-2). Interestingly, at Fox Hill ambient denitrification did not vary seasonally (Table

1-2, Fig. 1-4A). Other studies tracking salt marsh seasonal denitrification in vegetated and creek sediments have typically found that denitrification is lowest in the summer, and peaks in the spring and fall coinciding with increases in water-column nitrate (Eriksson et al. 2003; Hamersley and Howes 2005; Poulin et al. 2007), similar to the patterns we observed at Little Mussachuck. In vegetated sediments the seasonal activity of marsh plants can also affect denitrification. Root oxidation of the rhizosphere and leaching of nitrogen and carbon during plant senescence can stimulate coupled nitrification-denitrification, which may have been largely responsible for the peak in coupled activity we observed at both sites. During the growing season, competition for nitrogen can limit denitrification, which may possibly have affected spring and summer activity. In marshes with higher N loads however, microbes can outcompete plants for uptake of nitrogen (Teal and Howes 2000).

Denitrification rates measured in salt marshes tend to vary greatly, typically ranging  $0.01 - 14 \text{ mmol m}^{-2} \text{ d}^{-1}$  (Hamersley and Howes 2005; Hopkins and Giblin 2008). Ambient denitrification measured in our study was on the lower end of this range, with rates under  $1.0 \text{ mmol m}^{-2} \text{ d}^{-1}$ , and was lower compared to other studies conducted in Narragansett Bay marshes. Davis et al. (2004) measured net  $\text{N}_2$  flux at Fox Hill and reported net denitrification rates that were 10x greater than denitrification we captured at the same site. Because Davis et al. (2004) measured net  $\text{N}_2$  flux integrated down to 10cm, compared to the average depth of 4-5cm in our study, they likely captured more coupled denitrification occurring in the rhizosphere, which can be responsible for a large majority of denitrification in marsh platform sediments (Koop-Jakobsen and Giblin 2010). Our study likely underestimates total ambient

denitrification in marsh sediments because we captured surface activity only. Caffrey et al. (2007) also measured net  $N_2$  fluxes in vegetated sediments of a Narragansett Bay marsh, but in contrast to Davis et al. (2004), they measured activity denitrification only at the surface. Assuming that N fixation rates were low compared to denitrification in their net  $N_2$  fluxes (as seen in additional studies presented in Chapters 2 and 3), the net denitrification measured by Caffrey et al. (2007) were twice as high as those we found.

### ***Ambient Direct Denitrification and Coupled Nitrification-Denitrification***

A significant advantage to using the IPT method is that we were able to distinguish ambient rates of direct denitrification ( $D_w$ ) from coupled nitrification-denitrification ( $D_n$ ). This provided us with insight regarding the importance of tidal nitrate versus sediment nitrification in causing ambient denitrification to differ between sites. In general, higher direct denitrification was responsible for elevated ambient rates at Little Mussachuck in the fall, whereas in the spring, higher direct and coupled activity at Little Mussachuck were duly responsible (Fig. 1-3).

Over the annual cycle, direct denitrification was generally higher at Little Mussachuck, with very low or negligible activity at Fox Hill. The differences over time and between sites were tightly linked to water-column nitrate concentrations, indicating that direct denitrification was largely controlled by tidal nitrate availability. By comparing the magnitude of difference between sites in annual mean nitrate levels and direct denitrification, we also observed a proportionally greater response in direct denitrification to nitrate availability at Little Mussachuck (i.e. nitrate was only 4x

higher at Little Mussachuck than Fox Hill, but direct denitrification was 10x higher). This increased response at Little Mussachuck could be due to higher denitrification capacity or less competition for nitrate with plants, algae, or other nitrate-reducing microbes. As we will discuss later (see section on *Denitrification Capacity*), higher rates of denitrification capacity ( $D_{Total}$ ) measured at Little Mussachuck indicate that marshes with long-term N enrichment can develop a greater capacity for denitrification.

Similar to seasonal trends in general ambient denitrification, direct denitrification was relatively low at both sites throughout the spring and summer and peaked in the fall. Though nitrate availability seemed to be the main controlling factor for direct denitrification, temperature-limitation was also important, resulting in somewhat diminished activity in November and December even though nitrate levels were high. Other studies in temperate marshes have documented low temperatures to be limiting, shifting denitrification rates from being nitrate-limited to temperature-limited during cold seasons (Kaplan et al. 1977; Kaplan et al. 1979; Poulin et al. 2007). Wintertime denitrification may not be unimportant however. In a Canadian marsh overall denitrification remained at comparatively modest levels during the winter at temperatures as low as 2°C. Furthermore, high nitrate availability stimulated seasonally higher rates of direct denitrification in the winter, even though overall denitrification was at a seasonal low (Poulin et al. 2007). Therefore it is possible that nitrate removal via direct denitrification could be important during the winter in temperate regions, though this requires further investigation.

In contrast to direct denitrification, coupled nitrification-denitrification ( $D_n$ ) was similar between the two marshes, with the exception of May and June 2012 when rates were approximately 2x higher at Little Mussachuck (Fig 1-2C). We also did not observe significant seasonal variation at either site, though Little Mussachuck had a greater range in rates than Fox Hill owing to higher maximum activity. Coupled nitrification-denitrification is influenced by labile carbon availability, plant rhizosphere oxidation, and tidal inundation and drainage dynamics (Risgaard-Petersen and Jensen 1997). Although sediment % C was higher at Little Mussachuck than Fox Hill, SOD rates were nearly identical at both sites, indicating that labile carbon availability was also similar (see *Site Characterization* in the *Methods* section). In addition, the lack of a large difference in aboveground plant biomass and tidal flooding suggest that conditions that influence nitrification were likely similar at both marshes. We observed a seasonal peak in coupled nitrification-denitrification at both marshes in September, which could be due to senescence of the plants, causing roots to leach carbon and nitrogen into the sediments (Hopkinson and Giblin 2008). The higher activity measured at Little Mussachuck in late spring may be linked to enhanced root oxidation of the sediment during a time when root growth is at a maximum (Hines et al. 1989). This does not explain, however, why similarly augmented rates of coupled denitrification were not also seen at Fox Hill in late spring.

Ammonium uptake and production in the cores during incubations were very strongly linked to coupled nitrification-denitrification, suggesting that the ammonium in the water-column and produced within the sediment was ultimately reduced to  $N_2$

gas (Fig. 1-5B). Some ammonium could have been directly reduced to N<sub>2</sub> gas via anammox, though this is likely to be minimal. In recent years, some studies have examined the importance of anammox in marine and wetland systems. In organic rich coastal sediments, anammox comprises a small percentage (less than 10%) of total N<sub>2</sub> production (Engström et al. 2005). Less is known about the role of anammox in salt marshes, but a study in a New England marsh found that anammox only accounted for less than 3% of total N<sub>2</sub> production in fertilized and unfertilized vegetated sediments (Koop-Jakobsen and Giblin 2010).

In comparing the relative importance of coupled versus direct denitrification, annual coupled nitrification-denitrification dominated at both sites. At Fox Hill where tidal N concentrations were low, annual direct denitrification ( $0.02 \pm 0.01 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) comprised only 4% of annual measured N<sub>2</sub> production, with coupled activity ( $0.33 \pm 0.03 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) comprising the remaining 96% (Fig. 1-3). At Little Mussachuck direct denitrification ( $0.16 \pm 0.06 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) played a more significant role, comprising 28% of annual denitrification due to increased nitrate availability from higher tidal N concentrations. Annual coupled denitrification at Little Mussachuck ( $0.41 \pm 0.7 \text{ mmol m}^{-2} \text{ d}^{-1}$ ) still dominated net N<sub>2</sub> production at 72%. During the fall when nitrate concentrations were highest, direct denitrification significantly increased at both marshes. Though coupled denitrification continued to dominate fall seasonal rates, direct denitrification increased from being negligible to comprising 14-17% of ambient denitrification during fall months. At Little Mussachuck direct denitrification played a more important role in overall activity, accounting for 31-59% of ambient rates in the fall season. Previous studies have found that coupled denitrification is

typically favored in marshes and marine vegetated sediments due to the high organic content and root oxidation that fuels nitrification (Risgaard-Petersen and Jensen 1997; Hamersley and Howes 2003; Hamersley and Howes 2005; Koop-Jakobsen and Giblin 2010).

A few other studies have also measured the impact of N enrichment on direct and coupled denitrification separately. Koop-Jakobsen and Giblin (2010) quantified summertime rates of direct, coupled, and rhizosphere denitrification *in situ* in heavily fertilized and reference marshes in Plum Island Sound, MA. Under inundated conditions at high tide, they found that direct denitrification rivaled coupled nitrification-denitrification in the surface sediments of the reference marsh exposed to average tidal nitrate concentrations of 7  $\mu\text{M}$ . With intense long-term fertilization resulting in tidal nitrate levels of ~70-130  $\mu\text{M}$ , direct denitrification increased nearly 20-fold, comprising 94% of surface denitrification. In creek sediments and vegetated platforms of a highly N enriched Venice Lagoon marsh in Italy, direct denitrification tended to account for the majority of denitrification, particularly in the early fall when nitrate concentrations peaked (Eriksson et al. 2003). Hamersley and Howes (2005) found that coupled nitrification-denitrification dominated in a fertilized Cape Cod marsh, comprising 72% of denitrification in tidal creek sediments. Nowicki et al. (1999) also found that coupled nitrification-denitrification comprised the majority of  $\text{N}_2$  production in a Cape Cod marsh. Interestingly, while Nowicki et al. (1999) did not find any relationship of denitrification to groundwater N loading, Hamersley and Howes (2005) observed higher rates of coupled denitrification in heavily fertilized compared to unfertilized vegetated plots in another Cape Cod marsh.

From the results of these studies in addition to our current study, we can conclude that in organic rich sediments coupled nitrification-denitrification is favored as the main pathway for nitrate reduction to  $N_2$  gas, but that this can change as anthropogenic N loading increases and consequently enhances direct denitrification. However, the role of direct denitrification on the marsh platform in removing nitrate can be substantially limited. For instance, removal of tidal nitrate is limited to occurring during high tides when the marsh surface is inundated. Little Mussachuck and Fox Hill are inundated 23-25% of the day, with the exception of the neap tide during the 3<sup>rd</sup> quarter moon when the low marsh does not flood at either site. In Plum Island Sound, where the marsh platform in the *Spartina patens* zone is flooded for 12% of the day, Koop-Jakobsen and Giblin (2010) showed that the daily rates (accounting for lack of inundation at low tide) of direct denitrification, and hence its overall importance, were substantially diminished compared to hourly rates measured during high tides. Additionally, denitrification in the tidal creeks of the same marsh was much higher likely owing to longer exposure time to added fertilizer nitrate in the tidal waters.

### ***Denitrification Capacity***

In addition to distinguishing rates of ambient direct and coupled denitrification, we measured the denitrification capacity of marsh sediments in conditions of extremely high nitrate concentrations ( $\sim 160\mu M$  above ambient levels). With an essentially unlimited nitrate supply, denitrification capacity ( $D_{Total}$ ) was much higher at both marshes than ambient denitrification of tidal nitrate, often by an order of



magnitude (Fig. 1-4). Even during the late fall and early spring when activity markedly decreased at both marshes (likely due to cold temperatures), denitrification capacity was still 3-11x higher than respective ambient rates. Substantially elevated direct denitrification of the added  $^{15}\text{NO}_3^-$  tracer accounted for the surge in nitrate reduction activity. Additionally, the total capacity for denitrification was greater at the high N marsh, Little Mussachuck, compared to the low N marsh, Fox Hill, with the exception of cold months when rates were low at both sites. Koop-Jakobsen and Giblin (2010), using the IPT method to measure denitrification in marshes of Plum Island Sound, MA, also found that denitrification increased by more than an order of magnitude with the availability of high concentrations of the  $^{15}\text{NO}_3^-$  tracer. Similar to our findings in Little Mussachuck, denitrification capacity was higher in the creek banks of the fertilized marsh, compared to the unfertilized marsh. However, in the high marsh platform, which was exposed to tidal waters for only a fraction of the day, they did not find differences in the capacity for denitrification, whereas in this study we observed elevated denitrification capacity in the more tidally inundated low marsh. Wigand et al. (2004) used denitrification enzyme assays (DEA) to measure the denitrification potential of Narragansett Bay salt marshes in relation to modeled N loading and found a positive relationship in high marsh sediments, but no relationship in low marsh sediments. The DEA measurements of marshes with high N loads yielded potential rates that were two orders of magnitude larger than those we found in Little Mussachuck. This may be due to methodological differences, especially considering that sediments are slurried and incubated under ideal, non-limiting conditions for the DEA method, which may have enhanced denitrification potentials.

In addition to measuring ambient nitrate in estuarine waters adjacent to the marshes, we also monitored nitrate uptake in the sediments and overlying water during incubations. It is important to note that the nitrate concentrations we measured includes ambient tidal nitrate as well as the added  $^{15}\text{N}$  nitrate tracer, so we expected that overall uptake would be linked to total denitrification of  $^{14}\text{N}$  and  $^{15}\text{N}$ . However, we did not see a relationship between denitrification capacity and nitrate uptake during the incubations, nor were there any clear seasonal trends over the annual cycle (Figs. 1-4B and 1-5A). Uptake was higher at Little Mussachuck compared to Fox Hill in the fall, concurring with the greatest direct denitrification rates at Little Mussachuck. In addition, uptake rates were higher than measured denitrification. We speculate that other processes also contributed to nitrate uptake, such as dissimilatory nitrate reduction to ammonium (DNRA) activity or plant uptake. DNRA can be important in aquatic systems and is favored in labile carbon-rich sediments under nitrate-limiting conditions (Burgin and Hamilton 2007), though low rates of ammonium production in our cores indicate that DNRA was not likely very high. Another possibility is uptake by plant roots and rhizomes.

### ***Nitrate Removal***

The capacity for salt marshes to intercept and remove anthropogenic nitrate has been of great interest to coastal researchers and managers because marshes can potentially process, store, and remove large quantities of nitrogen (e.g. (Valiela et al. 1973; Drake et al. 2009; Brin et al. 2010)). In marshes exposed to high N loads, high rates of denitrification can potentially account for a significant portion of total

nitrogen retention and removal (Teal and Howes 2000). Based on our findings, increased N enrichment from estuarine waters does seem to stimulate marsh platform denitrification and the removal of excess nitrogen, though this is subject to seasonal conditions, tidal flooding, and other factors. While the low marsh accounts for a portion of the processing of water-column nitrogen, the creek bank and creek bottom sediments likely remove a larger amount of nitrogen via denitrification due to longer exposure to tidal waters and higher reported rates of denitrification (Kaplan et al. 1979; Koop-Jakobsen and Giblin 2010). Although this study focused specifically on the removal of nitrate from tidal waters, the marshes likely receive additional anthropogenic N inputs from groundwater and surface runoff. The landward boundary of the marshes and the creek bottoms likely intercept the majority of groundwater flow and therefore denitrification in these zones have the greatest potential for groundwater nitrogen removal (Howes et al. 1996).

Generally, pristine marshes with low N inputs tend to balance external N inputs with removal, burial, and plant uptake (Teal and Howes 2000). The low rates of denitrification we observed at Fox Hill illustrate that the role of gaseous losses of nitrogen is relatively small in unimpacted marshes. As N inputs increase, denitrification in marshes tends to remove more N, though overall importance of a marsh in removing N from an adjacent estuary is also dependent on the ratio of marsh to estuarine area and tidal regime (Nixon 1980).

In an attempt to quantify N removal at our study sites, we estimated the percent of tidal DIN removed by denitrification that potentially occurred in the low marsh per unit area (Table 1-3). Based on the duration of flooding and the height of the water

column measured using the HOBO data loggers and the DIN concentrations measured bi-monthly in the surface tidal water (Appendices A-9 and A-11), we calculated the average amount of DIN in tidal waters that flooded the short *S. alterniflora* zone on a monthly basis. At Little Mussachuck we estimated that an average of  $64.8 \pm 13.7$  mmol DIN m<sup>-2</sup> mo<sup>-1</sup> is brought into the low marsh via tidal flooding, compared to  $29.7 \pm 6.5$  mmol DIN m<sup>-2</sup> mo<sup>-1</sup> at Fox Hill. Using the ambient denitrification rates measured monthly, we calculated that, under flooding conditions during high tides, denitrification in the low marsh could remove an average of  $2.0 \pm 0.3$  mmol DIN m<sup>-2</sup> mo<sup>-1</sup> at Little Mussachuck and  $1.4 \pm 0.1$  mmol DIN m<sup>-2</sup> mo<sup>-1</sup> at Fox Hill. On a per month basis the % DIN removed from flooding waters in the low marsh ranged from 1 – 13% at Little Mussachuck and 2 – 31% at Fox Hill. Averaged over the annual cycle, % N removal was estimated to be 5% at Little Mussachuck and 12% at Fox Hill. We should note that we excluded June 2012 from Little Mussachuck from this analysis, because high rates of denitrification coincided with low nutrient levels that month, resulting in an anomalous 97% removal (% N removal was < 13% in all other months).

These estimates indicate that although low marsh N removal is relatively modest at both sites, it is not insignificant, especially considering that the low marsh is flooded (and has access to tidal DIN) only ~6 hours per day. As mentioned previously, other areas of the marsh may have an enhanced potential for N removal via denitrification, such as the creek banks and bottoms. Other studies have shown that the total N removal at the marsh ecosystem level is substantial. For example, Drake et al. (2009) found that in a fertilized marsh (with tidal nitrate concentrations of 84-96

μM) in Plum Island Sound, MA, 50-60% of nitrate inputs were processed and removed. In a fertilization study of a Cape Cod marsh, in which fertilizer was regularly applied directly on the marsh platform, 60-80% of nitrogen was intercepted and removed (Valiela et al. 1973). Compared to the Plum Island Sound marshes, which receive elevated N inputs via tidal waters and are limited by exposure time at high tide, the direct loading of fertilizers onto the marsh surface may have provided a greater opportunity for denitrifiers to remove the added nitrogen. Interestingly, decades later at the same Cape Cod marsh that had been continually fertilized on a long-term basis, the interception and processing of nitrogen increased to over 93% (Brin et al. 2010), indicating that prolonged exposure to high N enrichment likely enhanced N cycling. The higher denitrification capacity rates we observed at Little Mussachuck in this study also provide evidence to support this hypothesis.

Although we found evidence for an enhanced capacity for denitrification with N enrichment, the similarly low % N removed between the two marshes indicate that increased denitrification does not necessarily equate to an increased proportion of N removed per unit area of marsh. It is possible that the stimulation of denitrification serves to keep pace with increasing N inputs or may be overwhelmed, resulting in less efficient removal of N via marsh platform denitrification with N enrichment. At the ecosystem level, however, factors such as tidal flooding dynamics, seasonal temperature, and the ratio of marsh area to estuary area will greatly influence the overall proportion of estuarine N removed by marshes.

## ***Conclusion***

Overall, the results of this study illustrate some key consequences of nutrient enrichment on the denitrification and capacity for removal of anthropogenic N in salt marshes. On short time-scales denitrification is typically a nitrate-limited process and increasing N inputs resulted in subsequent increases in overall and direct denitrification. Because coupled nitrification-denitrification was similar between marshes with varying tidal N regimes yet total ambient denitrification was higher annually at the high N marsh, we conclude that increased tidal nitrate availability (as opposed to sediment carbon availability) stimulated denitrification at the N enriched marsh. Narragansett Bay, similar to other temperate estuaries, typically experiences strong seasonal changes in nitrogen concentrations, which had a strong seasonal impact on direct denitrification in the marshes we studied. This short-term response of denitrification to nitrate availability generally yields higher rates in the autumn and spring, with the lowest activity in the summer. In the winter, the response of denitrifiers to high nitrate availability may diminish when low temperatures limit microbial activity, and should be examined in future studies to better understand potential microbial nitrogen removal in the winter. Long-term exposure to high nitrate concentrations was likely responsible for an increase in the denitrification capacity of the surface marsh platform, indicating a shift in the microbial ecology of the marsh to process an increased abundance of nitrate. Though we observed an overall increase in denitrification linked to N fertilization, the proportion of DIN removed from the overlying water-column by the low marsh platform was relatively modest at both sites. This indicates that although N enrichment may stimulate denitrification on the marsh

platform, the total percentage of tidal N denitrified per unit area marsh does not necessarily increase as well. Other studies, however, have found significantly enhanced N removal in long-term fertilized marshes. Based on our findings, it is possible that the stimulation of denitrification by N enrichment may play a role in enhancing the capacity for marsh N removal at the ecosystem level.

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Table 1-1. Comparison of various parameters measured (mean values  $\pm$  SE) at Little Mussachuck marsh (LMK) and Fox Hill marsh (FOX). Ambient tidal water nitrogen concentrations of nitrate + nitrite ( $\text{NO}_{3+2}^-$ ), ammonium ( $\text{NH}_4^+$ ), and total dissolved inorganic nitrogen (DIN) represent averages samples collected 2x monthly (June 2011 to June 2012, excluding January, February, and March 2012;  $n = 19$ ). Aboveground end-of-season biomass (grams dry weight of live plant material;  $n = 6$ ) represents the biomass of *Spartina alterniflora* and *Spartina patens* measured in the low marsh. Average percentages of sediment carbon (C) and nitrogen (N), and C:N ratios ( $n = 3$ ) and percent organic matter ( $n = 5$ ) were analyzed in the top 15cm of sediment cores collected from the low marsh. Sediment oxygen ( $\text{O}_2$ ) demand was measured and averaged from bi-monthly incubations over an annual cycle ( $n = 19$ ). Tidal flooding was measured at both marshes over two lunar cycles from 8/24/12 to 10/21/12. Mean high water represents the water height above the low marsh surface and was averaged from peak water heights during peak high tides (2 days before through 2 days after full and new moon phases;  $n = 37$ ). The number of flooding events per month, as well as the average time period of each flooding event and the total time the low marsh was flooded each month is shown. Asterisks indicate significant differences between sites (t-tests,  $p < 0.05$ ).

Site	Ambient Tidal Water Nitrogen (Annual Average) ( $\mu\text{M}$ )			Aboveground Peak Productivity ( $\text{g dw m}^{-2}$ )	Sediment % C *	Sediment % N *	C:N	Sediment Oxygen Demand ( $\text{mmol m}^{-2} \text{d}^{-1}$ )	Tidal Flooding in Low Marsh			
	$\text{NO}_{3+2}^-$ *	$\text{NH}_4^+$ *	DIN *						Mean High Water (m)*	# Flooding Events per Month	Average Period of Flooding	Total Time Flooded (hrs/month)
LMK												
High N	$8.2 \pm 2.0$	$4.7 \pm 1.1$	$12.9 \pm 2.9$	$407 \pm 5$	$20.7 \pm 1.1$	$1.2 \pm 0.1$	$18.0 \pm 0.6$	$53.3 \pm 3.6$	$0.19 \pm 0.02$	29	$3.0 \pm 0.2$	85
FOX												
Low N	$2.5 \pm 0.7$	$1.7 \pm 0.3$	$4.2 \pm 0.9$	$475 \pm 40$	$15.3 \pm 0.6$	$0.9 \pm 0.1$	$16.9 \pm 0.5$	$46.9 \pm 2.9$	$0.23 \pm 0.01$	37	$2.7 \pm 0.1$	100

Table 1-2. Statistical results for site characterization and denitrification (IPT) incubations. Statistics tests used, F ratios and degrees of freedom (test and model error), and significance ( $p$ -values) of tests for differences between sites and among seasons. An asterisk on the  $p$ -value denotes significance (where  $p \leq 0.05$ ). Dashes represent results that are not applicable or available based on the specific test run.

Variable	Test Used	F Ratio (Degrees of Freedom)			<i>p</i> -value			Data
		Site	Season	Interaction	Site	Season	Interaction	Transformation
<u>SITE CHARACTERIZATION DATA</u>								
Ambient Tidal NO <sub>3+2</sub> <sup>-</sup>	Friedman Test	7.96 (1, 36)	32.78 (2, 36)	-	0.008*	<0.0001*	-	Log (Natural)
Ambient Tidal NH <sub>4</sub> <sup>+</sup>	Two-way ANOVA	10.58 (1, 34)	24.15 (2, 34)	1.59 (2, 34)	0.002*	<0.0001*	0.22	
Ambient Tidal DIN	Friedman Test	10.84 (1, 36)	44.97 (2, 36)	-	0.002*	<0.0001*	-	
Aboveground Biomass	One-way ANOVA	0.49 (1, 10)	-	-	0.50	-	-	
Sediment %C	One-way ANOVA	17.88 (1, 28)	-	-	0.0002*	-	-	Log (Natural)
Sediment %N	One-way ANOVA	8.27 (1, 28)	-	-	0.008*	-	-	
Sediment C:N	One-way ANOVA	2.19 (1, 28)	-	-	0.15	-	-	
Sediment O <sub>2</sub> Demand	Two-way ANOVA	2.80 (1, 34)	3.18 (2, 34)	0.76 (2, 34)	0.10	0.05*	0.48	
Mean High Water	Welch ANOVA	5.26 (1, 61)	-	-	0.03*	-	-	Square Root
Average Period of Flooding	Welch ANOVA	0.77 (1, 88)	-	-	0.38	-	-	Square Root
<u>IPT INCUBATION DATA</u>								
Ambient Tidal NO <sub>3+2</sub> <sup>-</sup>	Friedman Test	19.16 (1, 16)	22.93 (2, 16)	-	0.0005*	<0.0001*	-	Log (Natural)
Sediment O <sub>2</sub> Demand	Two-way ANOVA	0.02 (1, 14)	5.25 (2, 14)	0.23 (2, 14)	0.88	0.02*	0.80	
Ambient Denitrification ( <i>D</i> <sub>14</sub> )	Two-way ANOVA	4.90 (1, 14)	0.36 (2, 14)	0.36 (2, 14)	0.04*	0.70	0.71	
Ambient Direct Denitrification ( <i>D</i> <sub>w</sub> )	Friedman Test	22.07 (1, 16)	9.86 (2, 16)	-	0.0002*	0.002*	-	
Ambient Coupled Denitrification ( <i>D</i> <sub>n</sub> )	Two-way ANOVA	0.20 (1, 14)	0.45 (2, 14)	0.04 (2, 14)	0.67	0.65	0.96	Log (Natural)
Denitrification Capacity ( <i>D</i> <sub>14</sub> + <i>D</i> <sub>15</sub> )	Two-way ANOVA	15.30 (1, 14)	4.5 (2, 14)	0.79 (2, 14)	0.002*	0.03*	0.47	
NH <sub>4</sub> <sup>+</sup> Production/Uptake in Cores	Two-way ANOVA	0.01 (1, 14)	3.49 (2, 14)	0.54 (2, 14)	0.94	0.06	0.59	Log (Natural)
NO <sub>3+2</sub> <sup>-</sup> Production/Uptake in Cores	Two-way ANOVA	0.33 (1, 14)	2.62 (2, 14)	1.48 (2, 14)	0.57	0.11	0.26	

Table 1-3. Removal of dissolved inorganic nitrogen (DIN) by ambient denitrification in the low marsh at Little Mussachuck (LMK) and Fox Hill (FOX) on a monthly basis. DIN loadings, denitrification rates, and % DIN removed were calculated based on ambient surface DIN concentrations in the tidal water, ambient denitrification ( $D_{14}$ ) occurring during high tides, the total duration of flooding at high tide in the low marsh, and the volume of water flooding the low marsh per month. An average from all 10 month is shown for each calculated parameter, including standard error (s.e.). \*This average excludes June 2012 at Little Mussachuck, which had an anomalously high % DIN removed – if included the monthly average would be  $14.5 \pm 9.3\%$ .

Year	Month	DIN Loading to Low Marsh (mmol m <sup>-2</sup> mo <sup>-1</sup> )		DIN Denitrified at High Tide (mmol m <sup>-2</sup> mo <sup>-1</sup> )		% DIN Removed via Denitrification	
		LMK	FOX	LMK	FOX	LMK	FOX
2011	June	7.5	3.6	0.33	0.71	4.4	19.9
	July	21.1	21.6	1.52	1.68	7.2	7.8
	August	76.8	24.6	1.63	1.14	2.1	4.6
	September	79.3	18.2	2.89	2.22	3.6	12.1
	October	161.1	58.2	3.66	1.49	2.3	2.6
	November	151.3	63.8	2.22	1.28	1.5	2.0
	December	115.7	86.5	1.46	1.28	1.3	1.5
2012	April	7.7	7.9	1.01	1.77	13.1	22.3
	May	24.9	4.8	3.11	1.50	12.5	31.0
	June	2.6	8.1	2.55	1.44	97.2	17.6
Monthly Average ( $\pm$ s.e.)		64.8 $\pm$ 13.7	29.7 $\pm$ 6.5	2.04 $\pm$ 0.33	1.44 $\pm$ 0.13	5.3 $\pm$ 1.5 *	12.2 $\pm$ 3.2

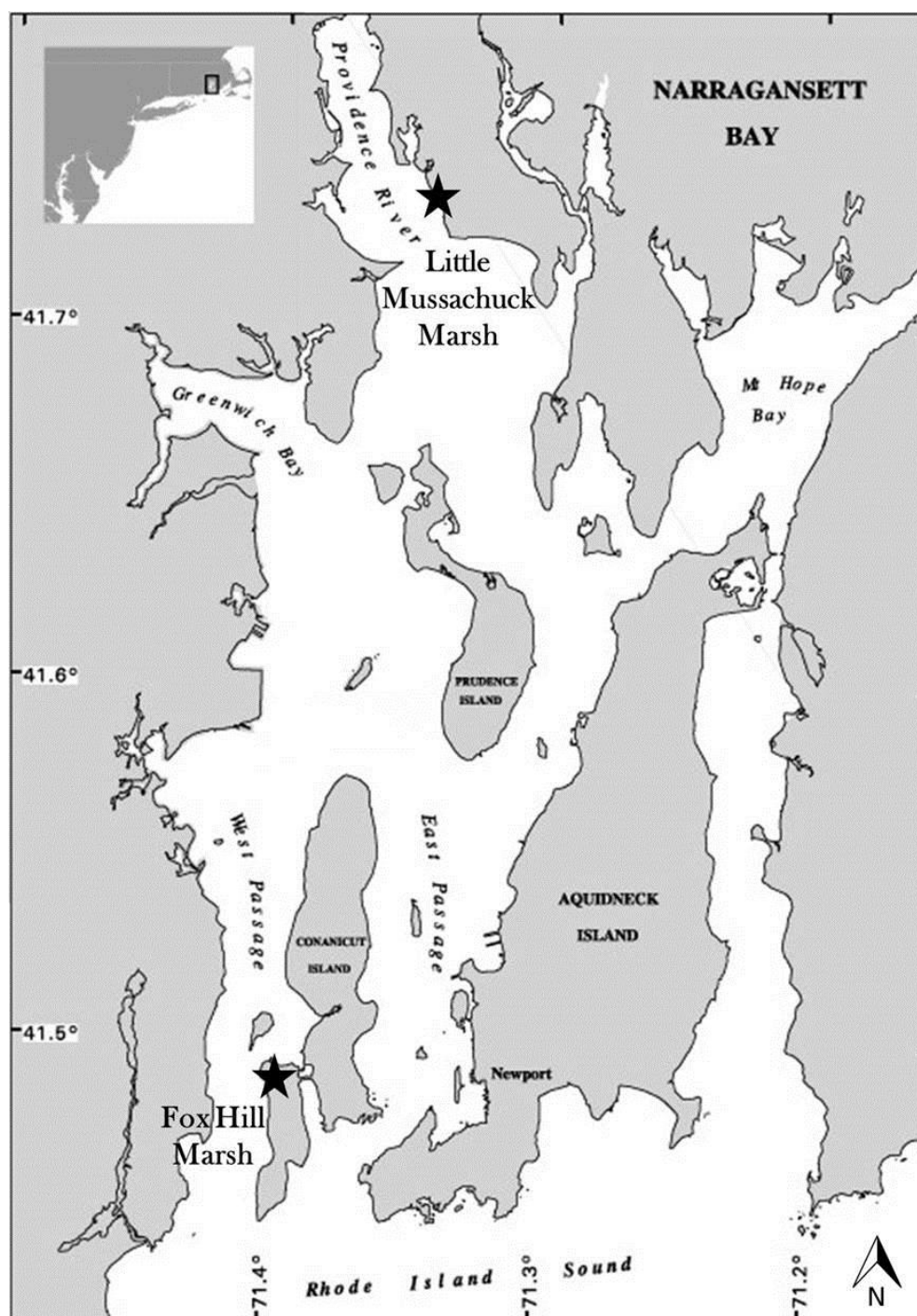


Figure 1-1. Locations of study sites in Narragansett Bay, Rhode Island. Map of Narragansett Bay courtesy of <http://www.gso.uri.edu/phytoplankton/>. Map data provided by RIGIS.



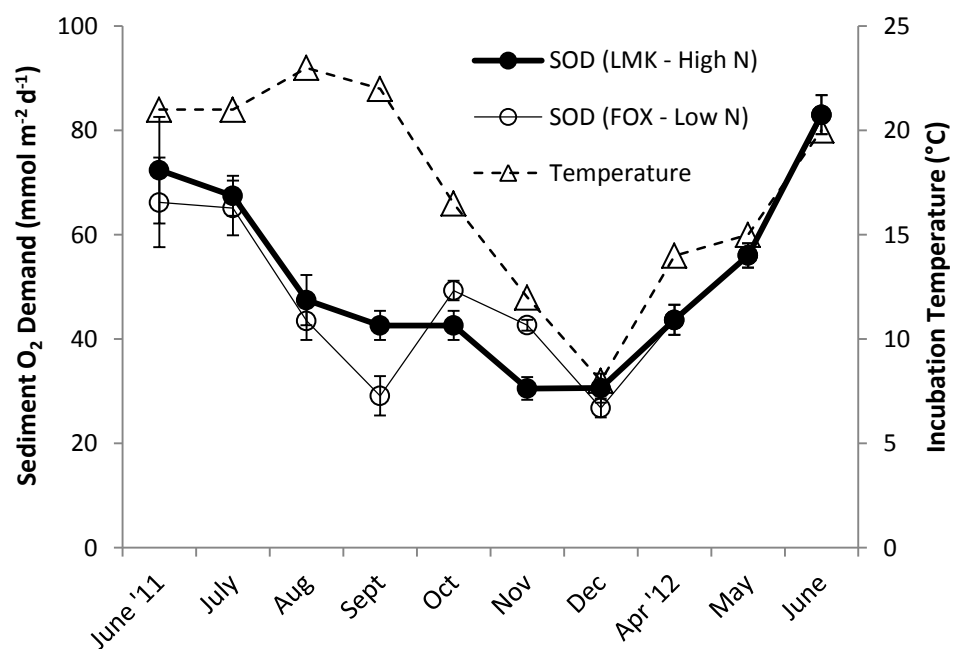


Figure 1-2. Sediment oxygen demand (SOD) measured monthly at two marshes with high and low nitrogen (N) inputs from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Monthly incubation temperatures were determined from ambient surface soil temperatures measured during the time of sediment core collection. Error bars represent standard error based on linear regressions.

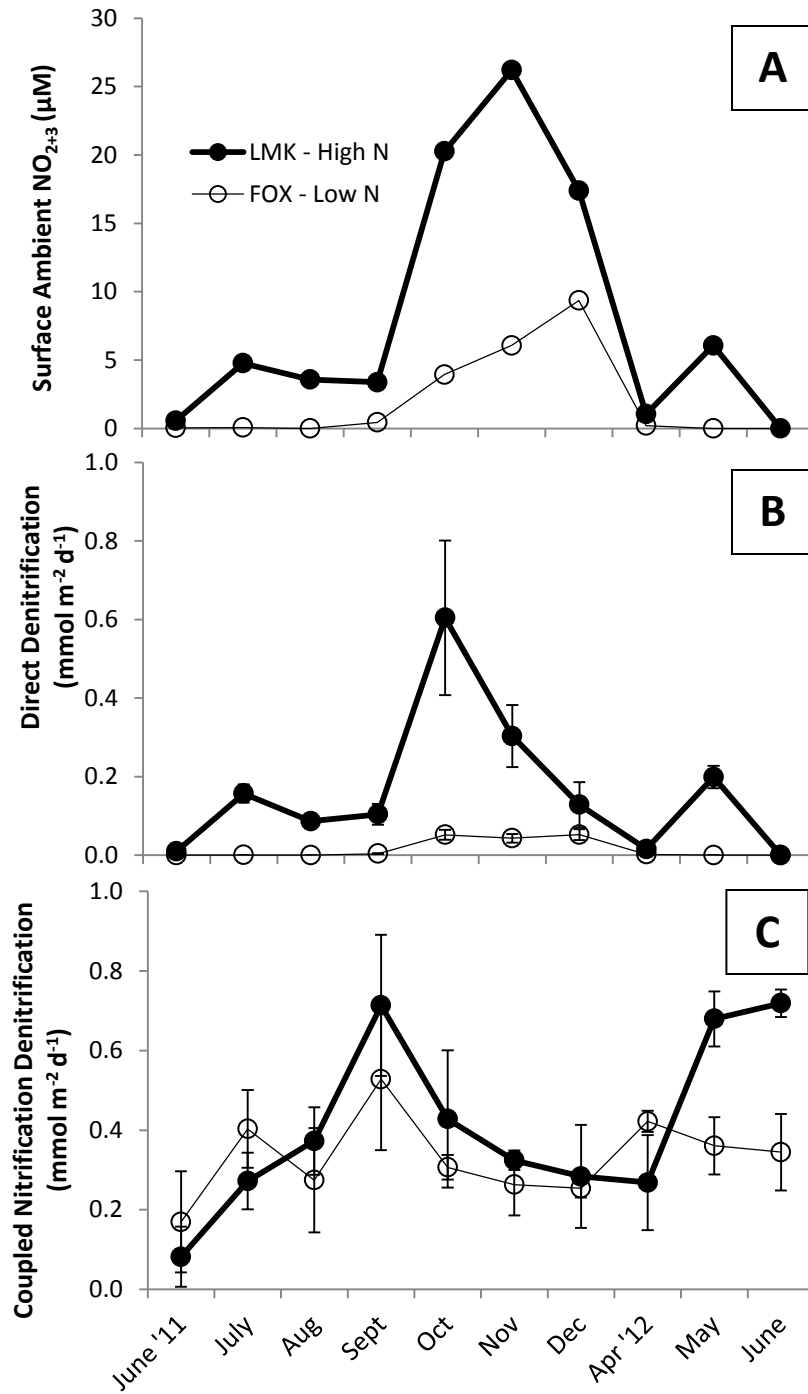


Figure 1-3. (A) Ambient nitrate-nitrite concentrations in the surface tidal water, (B) ambient direct denitrification ( $D_w$ ) of naturally-occurring nitrate in the overlying water, and (C) ambient coupled nitrification-denitrification ( $D_n$ ) of nitrate produced in the sediment, measured monthly at two marshes with high and low nitrogen (N) inputs from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Error bars represent standard error based on linear regressions.

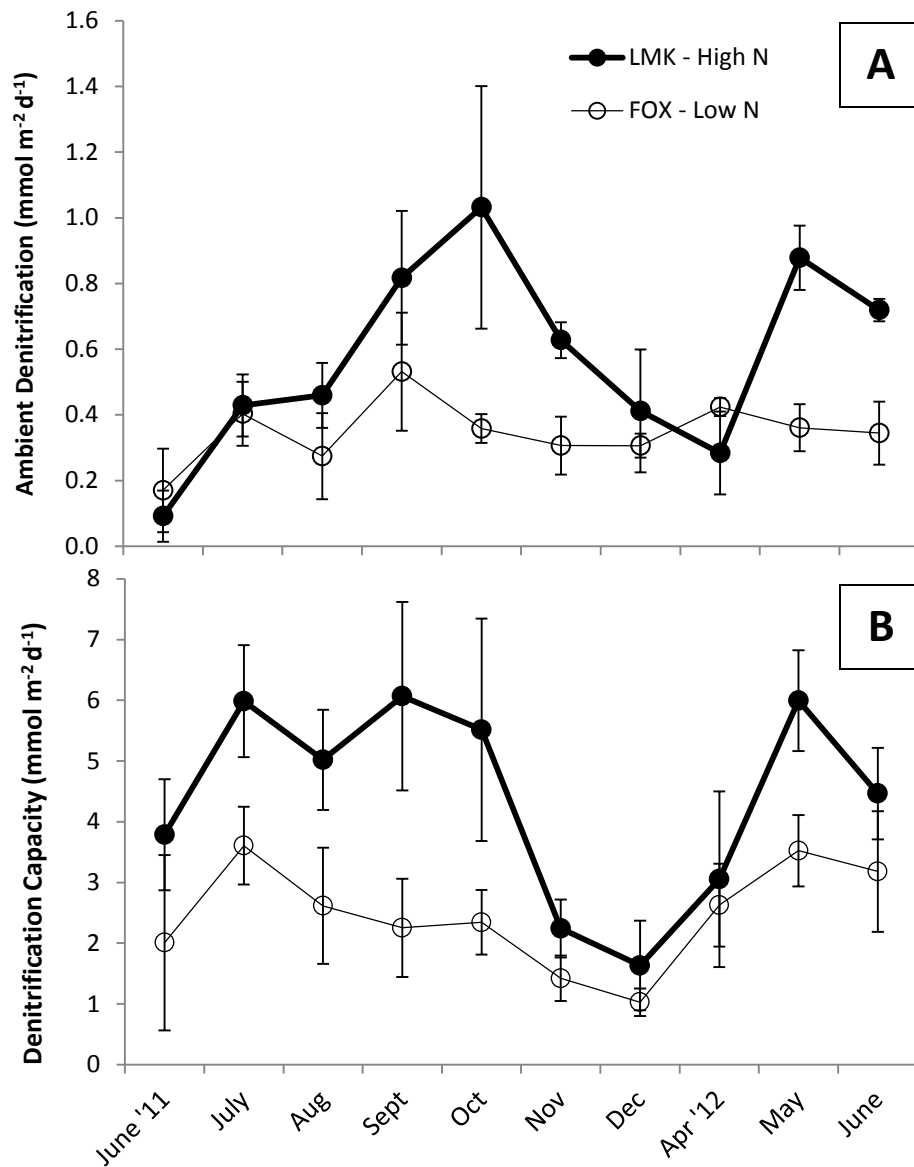


Figure 1-4. (A) Ambient denitrification ( $D_{14}$ ) of ambient nitrate present in tidal and porewater and (B) total denitrification capacity ( $D_{Total}$ ) of ambient nitrate plus the added  $^{15}\text{N}$ -nitrate tracer, measured monthly at two marshes with high and low nitrogen (N) inputs from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Error bars represent standard error based on linear regressions.

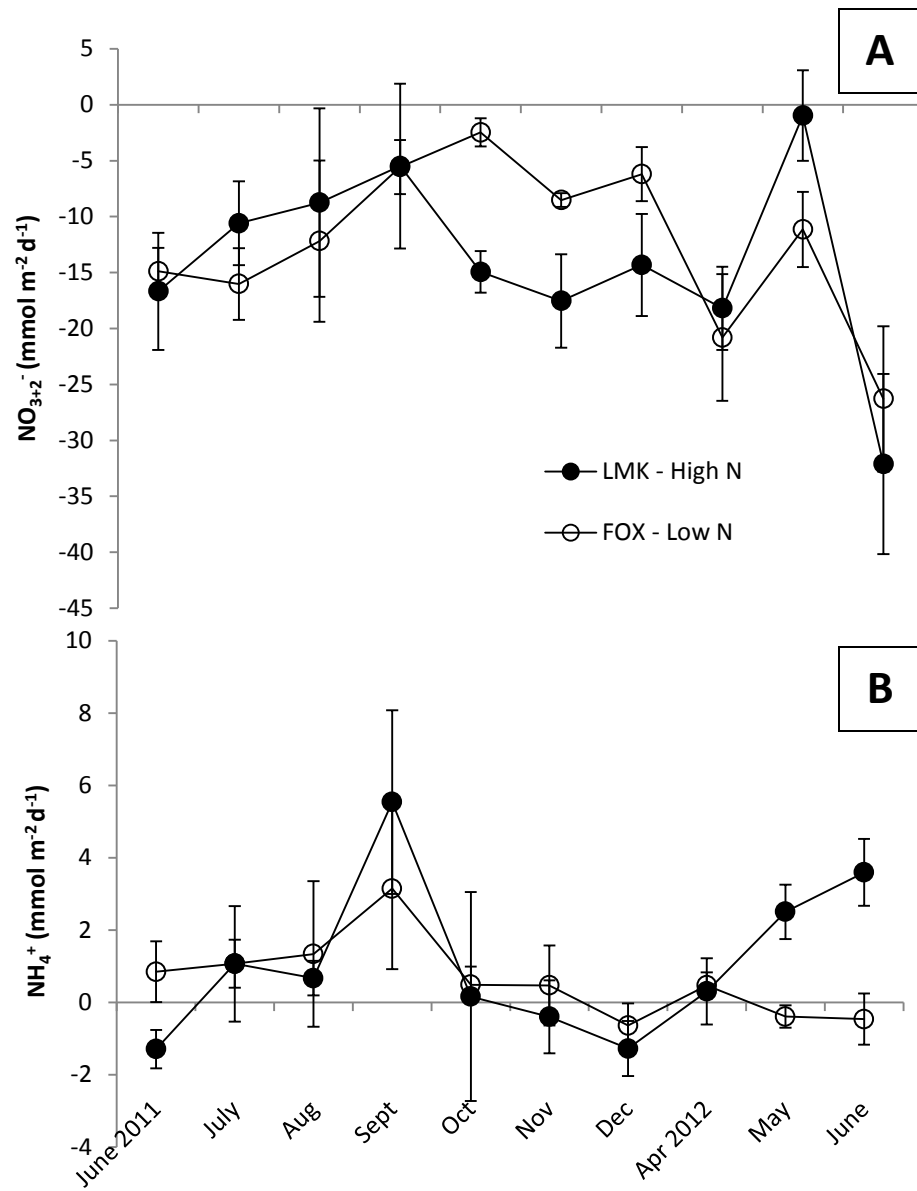


Figure 1-5. **(A)** Nitrate-nitrite and **(B)** ammonium uptake and production at the sediment-water interface measured monthly at two marshes with high and low nitrogen (N) inputs from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Negative values represent nutrient uptake in the sediments and positive values represent nutrient production. Error bars represent standard error based on linear regressions.

## CHAPTER 2

### PREFACE

# **NITROGEN FIXATION IN NEW ENGLAND SALT MARSHES: EXAMINING THE IMPACT OF NUTRIENT ENRICHMENT OVER AN ANNUAL CYCLE AND COMPARING INCUBATION METHODS**

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## CHAPTER 2

# NITROGEN FIXATION IN NEW ENGLAND SALT MARSHES: EXAMINING THE IMPACT OF NUTRIENT ENRICHMENT OVER AN ANNUAL CYCLE AND COMPARING INCUBATION METHODS

## ABSTRACT

Nitrogen (N) fixation in salt marshes has been the subject of decades of research, as it can serve as an important source of biologically available N that fuels marsh primary production. However, high anthropogenic N inputs to coastal ecosystems have the potential to significantly alter N fixation dynamics in salt marshes. While high N concentrations often suppress N fixation, several other factors such as carbon availability, temperature, and marsh plant dynamics can also control N fixation rates, and previous work has demonstrated varying responses of N fixation to N fertilization. Using the commonly employed acetylene reduction assay technique, we investigated the impact of long-term nutrient enrichment on N fixation in vegetated marsh sediments by comparing two salt marshes exposed to high and low tidal N concentrations in Narragansett Bay, Rhode Island. We examined how sediment N fixation varied over time by making monthly measurements over an annual cycle (excluding winter months) using whole sediment cores incubated *in vitro* under ambient, flooded conditions. Most studies in marshes have not used whole core measurements and instead generally relied on incubating relatively small sediment

samples. Therefore we also measured N fixation using bottle incubations of small samples of sediment “plugs” several times during the year, allowing for a direct comparison of incubation methods in salt marsh sediment N fixation. Although the sediment plug method yielded statistically higher rates of N fixation compared to whole cores, both methods indicated significantly lower rates of N fixation averaged over the annual cycle in the highly nutrient enriched marsh. We did not observe significant seasonal changes over an annual cycle in the intact core measurements, though N fixation visibly peaked in the early fall at both sites. We attributed this peak to the coupling of N fixation with enhanced root exudation of labile C during plant senescence, which has been previously documented in the vegetated sediments of salt marsh and seagrass systems. Because microbial carbon availability (indicated by sediment oxygen demand) in the intact cores was similar between the two sites, we concluded that the lower N fixation rates in the nutrient enriched marsh was due to suppression by higher concentrations of ammonium and nitrate + nitrite in tidal water and porewater. Despite inherent weaknesses in the two incubation techniques, which we discuss in detail, the likely suppression of N fixation by nutrient enrichment was nonetheless detectable over time using both methods.

## INTRODUCTION

Human activities have heavily impacted coastal waters worldwide, especially within the last several decades. Coastal eutrophication resulting from high inputs of nitrogen and phosphorus via sewage outflows and urban and agricultural runoff has resulted in the widespread degradation of many coastal ecosystems around the world (National Research Council 2000; Howarth et al. 2002). Because of its complex effects on ecosystem processes, high nutrient loading to coastal waters has resulted in various impacts that include severe and chronic low oxygen conditions, changes to community diversity and structure, an increase in the severity and frequency of algal blooms, and the loss of submerged aquatic vegetation (Nixon 1995; Vidal et al. 1999; Rabalais and Nixon 2002). The impact on salt marshes is of particular interest to coastal researchers and managers due to critical ecosystem services provided by marshes such as biogeochemical cycling of nutrients and providing important habitat for terrestrial and marine species (Teal 1962; Valiela and Teal 1979; Boesch and Turner 1984; Deegan and Garritt 1997; Craig and Crowder 2000).

In particular, the effects of high anthropogenic nutrient loading on the microbial-mediated cycling of nitrogen (N), the limiting nutrient in most coastal and salt marsh systems, have important implications for marsh plant communities and overall ecosystem productivity (Scott et al. 2007; Hopkins and Giblin 2008).

Nitrogen fixation, the process by which some photosynthetic, heterotrophic, and chemotrophic bacteria fix nitrogen gas ( $N_2$ ) into a biologically available form ( $NH_3$ ), typically serves as an important source of new nitrogen to stimulate plant growth in



salt marshes, particularly in newly establishing and oligotrophic marshes (Tyler et al. 2003). Our understanding of the impact of high N loading on marsh N fixation is not entirely straight-forward, especially because our understanding of the complex ecological controls on N fixation is still somewhat poor (Vitousek et al. 2002). Various abiotic (bottom-up) and biotic (top-down) factors such as labile carbon availability, inorganic nutrient availability (nitrogen, phosphorus, iron, and molybdenum), light, redox potential, salinity, and grazers contribute to controlling N fixation (Vitousek et al. 2002). While photosynthetic N-fixers are often limited by light availability and grazing, N fixation in the sediments is typically carbon-limited and closely coupled with plant root dynamics, as root exudates provide a source of carbon to N-fixers and N fixation provides nitrogen for plant growth (Carpenter et al. 1978; Welsh et al. 1996; De Souza and Yoch 1997; McGlathery et al. 1998). This complex relationship, however, is still not well-understood (Coleman 2008).

Dissolved inorganic nitrogen is also an ecologically important factor that directly and indirectly influences N fixation. High concentrations of ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) can directly control N fixation by suppressing activity of nitrogenase, the enzyme responsible for N fixation (e.g. Van Raalte et al. 1974; Carpenter et al. 1978; Dicker and Smith 1980b; Yoch and Whiting 1986). Marsh fertilization studies have reported lower rates of N fixation resulting from exposure to elevated N concentrations ( $>7\mu\text{M N}$ ; (e.g. Carpenter et al. 1978; Moseman-Valtierra et al. 2010), though one study found that this effect was reversible when low N conditions were restored (Bagwell and Lovell 2000). High N loading to marshes can also have indirect effects on N fixation. The impact of N fertilization on plant

dynamics and plant-microbial coupling, for instance, has the potential to indirectly alter rhizosphere N fixation and associated controlling factors. Studies reported that short-term N fertilization enhanced N fixation likely due to an increase in plant productivity and root exudation of carbon (Hanson 1977; Piceno and Lovell 2000). This trend, however, was not sustained during long-term exposure to high N concentrations (Piceno and Lovell 2000), indicating that the effects of N fertilization are dynamic.

To better understand the impacts of long-term high N loading on salt marsh N fixation, we compared activity of heterotrophic N-fixers in sediment cores collected from marshes with varying tidal N concentrations in Narragansett Bay, Rhode Island. With a long history of high anthropogenic N loading, in addition to the well-established gradient in nutrient concentrations along the length of the bay (Oviatt et al. 2002; Nixon et al. 2008; Oviatt 2008), Narragansett Bay is an excellent system in which to conduct such a comparison. Furthermore, we sought to characterize the impact of high N concentrations to heterotrophic N fixation on a seasonal basis in order to better understand the potential relationship of N fixation activity to seasonal changes in biotic and abiotic variables. The final goal of our study was to compare incubation methods of vegetated sediments using a method we developed to measure N fixation in intact sediment cores under flooded conditions, versus a more commonly employed “bottle incubation” using small samples of sediments (which we refer to as “sediment plugs”) in an oxygen-free atmosphere. The sediment plug method (or variations similar to it, such as creating “slurries” with seawater and sediment) is commonly used because it is relatively simple in design and execution, allows for

more replication of measurements, and provides a simple mechanism for measuring sediment N fixation at depth. However, in seagrass dominated systems it has been speculated that slurry and plug methods can overestimate N fixation rates by releasing labile carbon while breaking up roots, while in contrast whole core methods can underestimate rates if the attained acetylene saturation is low or patchy in the sediments (Patriquin and Knowles 1972; Capone 1988; Welsh et al. 1996; Hansen and Lomstein 1999; Welsh 2000). While a few comparisons among methods to measure N fixation have been tested in seagrass sediments, to date, no direct comparisons in vegetated salt marsh sediments have been made. To address this we measured N fixation using whole cores and bottle incubations of sediment plugs in tandem at various times over an annual cycle.

## **MATERIALS AND METHODS**

### ***Study Areas***

Narragansett Bay and the study sites for this study have been described in detail in Chapter 1 of this dissertation. In brief, Narragansett Bay is a well-mixed, phytoplankton-based estuary (328 km<sup>2</sup>) that spans a large length of the Rhode Island coastline (Nixon et al. 1995). A large majority of anthropogenic inputs enter near the head of the Bay, in a highly urbanized area, where riverine inputs and wastewater treatment outflows have resulted in a well-established north-south gradient in nutrient and phytoplankton concentrations (Nixon et al. 1995; Oviatt et al. 2002; Oviatt 2008).

Our study sites lie at opposing ends of the north-south nutrient gradient (Fig. 2-1). Little Mussachuck marsh (Barrington, RI; 4.4 hectares), located in the eutrophic Providence River Estuary, receives high nutrient inputs from tidal waters on an annual basis, particularly in the fall and winter months. In contrast, Fox Hill marsh (Jamestown, RI; 10.0 hectares) is located near the mouth of Narragansett Bay where the nutrient inputs from tidal waters remain fairly low year-round. Both sites exhibit vegetation zonation typical of New England salt marshes, with tall form *Spartina alterniflora* lining the creek bank, short form *S. alterniflora* covering the majority of the low marsh, and *Spartina patens* dominating the high marsh. Additional characteristics of the two marshes including aboveground peak biomass, marsh platform flooding, porewater dissolved inorganic nitrogen (DIN) concentrations, and sediment carbon (C), N, and organic matter content have been described in Chapter 1 (Table 1-1).

### ***Acetylene Reduction Assay***

To measure N fixation we used the acetylene reduction assay (ARA; Stewart et al. 1967). Because directly measuring the uptake and fixation of N<sub>2</sub> gas is difficult in an atmosphere composed of 80% N<sub>2</sub>, acetylene reduction is commonly used as a proxy measurement. Nitrogenase, the enzyme in diazotrophs responsible for converting N<sub>2</sub> gas into NH<sub>4</sub><sup>+</sup>, also reduces acetylene gas into ethylene. Following exposure to acetylene, the production of ethylene by diazotrophs is assumed to occur in a theoretical 3:1 ratio of moles of ethylene produced to moles of N<sub>2</sub> gas fixed (equivalent to a 3:2 ratio of ethylene produced to NH<sub>4</sub><sup>+</sup> produced). Only a few studies

in salt marshes have calibrated this ratio against direct measurements of N fixation in salt marshes, with one study confirming the theoretical ratio of 3:1 (ethylene produced to N<sub>2</sub> fixed) (Currin et al. 1996), another having found a ratio of 3:2 (Carpenter et al. 1978), and a third study in a brackish marsh reporting a ratio of 3.6:1 (DeLaune and Patrick 1990). Due to the costly and complicated methods to execute a calibration of the ratio, most published studies forgo the calibration and instead report results in ethylene produced or use the theoretical ratio of 3:1. Although the potential for this ratio to vary is an inherent weakness in the method, the ARA technique continues to be widely used in N fixation salt marsh research and is especially useful for comparative studies such as this one.

### ***Core Collection***

We collected intact sediment cores from the low marsh at both sites on a monthly basis from July 2011 to June 2012 to measure heterotrophic N fixation over an annual cycle. Winter months (January through March), when the marsh sediment is often frozen, were excluded from our monthly measurements. To measure N fixation using whole cores we extracted five sediment cores (10 cm diameter, 15 cm deep) at low tide within a 0.5 hectare area of each marsh. We collected cores in between plant shoots in order to exclude shoots in the cores. Though the cores were bare on the surface the sediments contained many roots and rhizomes and, at times, contained very small, budding shoots. Therefore we considered the sediment cores to be “vegetated”. The cores were collected by hammering core tubes with sharpened bottoms to depth and then carefully digging them out. We collected sediments within

the small bare patches scattered among the vegetation to exclude large plant shoots (which can create problematic gas bubbles) from our cores. Concurrent with core sampling, surface tidal water was collected adjacent to each marsh to use as overlying water during the incubations and to determine ambient nutrient concentrations. In August, September, November, and June we collected additional cores (concurrent with the sampling described above) to measure N fixation using small plugs of sediment. The smaller cores (5 cm diameter) were placed immediately adjacent to the larger cores, hammered to depth (15 cm) and dug out. Following collection, the sediments and tidal water were transported and stored at ambient surface soil temperatures in a temperature-controlled environmental chamber at the Graduate School of Oceanography. The larger cores were left to drain overnight (mimicking low tide), and the tidal water was filtered to remove particles greater than 0.2 microns. We measured heterotrophic N fixation in the larger cores, using the whole core method, the following day. The smaller cores (if collected during a specific month) were kept in the environmental chamber in an upright position to drain until they were incubated the next day to measure N fixation using the plug method.

### ***Core Incubations***

#### ***Whole Core Method***

The day following collection, we incubated the larger, intact sediments cores to measure N fixation. Immediately prior to beginning the incubation, we exposed the sediments to acetylene-amended seawater during a 2 hour “pre-incubation” in order to

ensure that the amended water would have adequate time to saturate the sediments. The goal of the pre-incubation was to eliminate any lag-time in ethylene production during our actual incubations due to the time required for the amended water to drain and diffuse into the sediments. Preliminary tests using a bromide tracer showed that a 2-hour pre-incubation resulted in the tracer-amended water penetrating the sediment to 6 cm depth (Appendix A-7). Because rates were calculated based upon a linear regression of measurements taken at several time points after the pre-incubation, any ethylene production during the pre-incubation did not affect the calculated rate. To amend the seawater, we bubbled 2 liters of tidal water collected from each marsh with acetylene gas for 1 hour to reach 100% saturation. The acetylene-saturated water was then added to an additional 18 liters of tidal water and gently mixed to create an approximately 10% solution of amended seawater. We kept the batches of seawater collected from each marsh separate in order to expose the sediments to site-specific tidal water during the incubations. For the pre-incubation, the cores were gently filled with amended tidal water, covered with floating Styrofoam caps to reduce gas loss, and the water slowly drained through a small opening in the bottom of the cores. The physical drainage helped to pull amended water into the sediments and was slow enough that the surface of the cores remained flooded throughout the entire pre-incubation.

At the end of the 2-hour pre-incubation, the remaining overlying water was siphoned off and replaced with freshly made acetylene-amended tidal water. The cores were filled completely (resulting in a water-column 43 cm deep) and capped with gas-tight lids fitted with sampling ports. Floating magnetic stirrer bars that were

placed mid water-column within the cores ensured mixing of the overlying water. We incubated the sediments for 7-8 hours and collected water samples throughout (every ~2.5 hours) to analyze for ethylene production and nutrient concentrations ( $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$ ). Overlying water samples were collected using a gravity flow-through system in order to replace any water sampled. Though the replacement seawater had been amended with acetylene, it is likely that a significant portion of the acetylene diffused out of the water over time.

We chose the maximum incubation time of 8 hours because longer ARA incubations can potentially overestimate N fixation rates due to the artificial increase of nitrogenase activity resulting from long-term acetylene exposure (Howarth and Marino 1988). However, the 8-hour incubation time does not likely allow the production of ethylene in the sediments to come to equilibrium with the overlying water. Therefore, at each time point following the collection of overlying water samples, we destructively sampled the porewater from the top 3-6 cm of one core from each marsh throughout the incubation, resulting in the eventual sacrifice of all the cores, one at each time point. This method, which measures subsurface N fixation, did not capture activity occurring deeper in the rhizosphere where significant N fixation has been shown to occur (e.g. Teal et al. 1979; Dicker and Smith 1980a). Although this method therefore likely underestimates total N fixation, it may provide a more accurate estimation of subsurface activity compared to slurry and plug methods that disturb the sediment structure. Because samples from the porewater-overlying mix in the whole cores captured more activity (Appendix A-8), we reported only the data



measured from the porewater-overlying mix and have not included data from the overlying water.

The porewater was sampled by using a large metal fork to gently break up the top layer of sediment and mix it with the overlying water. We then collected samples of the porewater-overlying water mix to analyze for ethylene production and nutrient concentrations. To measure the volume of porewater mixed into the overlying water, a known amount (3-5 mL) of concentrated bromide tracer was added to the water-column prior to mixing (Appendix A-1). Duplicate samples (6 mL) for bromide analysis were taken before and after destructive porewater sampling and stored at 4°C until analysis. To measure sediment oxygen demand, which is indicative of microbial C availability (see Ch. 1 *Methods* section), and to also ensure that the overlying water did not become hypoxic (<4 mg/L O<sub>2</sub>) we monitored oxygen concentrations throughout the incubation using a Hach HQ30 LDO probe inserted through a small, stoppered opening in the lids of the cores (Hach Company). Water temperature was also measured using the Hach probe. For ethylene production, we collected replicate samples of 50 mL each with a 10 mL headspace in serum bottles. Samples were fixed with 1 mL of zinc chloride, stoppered with rubber septa and sealed with aluminum crimp caps, and stored underwater up to a maximum of one week until analysis. We collected 60 mL of water for nutrient analysis, filtered the samples through 0.45 µM Whatman glass microfiber filters, stored in acid-washed polyethylene bottles, and frozen at -15°C until analysis. Two 6 mL samples for bromide analysis were taken before and after destructive porewater sampling and stored at 4°C.

### *Sediment Plug Method*

In August, September, November, and June we measured N fixation using small sections of sediment (plugs) to compare methods the day following the whole core incubations. We sectioned the top 5 cm of sediment into two depth layers (0-2 cm and 2-5 cm), and thoroughly homogenized the sediment sections. In November and June we added a third depth layer (5-10 cm) to our incubations. Duplicate subsamples 5 cm<sup>3</sup> in volume were subsequently placed into 40 mL vials, flushed with argon to prevent further exposing the anoxic sediment to oxygen during the incubations, and capped. To start the incubation we added 10 mL of acetylene gas and shook the vials, then briefly vented them to restore atmospheric pressure. The vials were incubated for 7-8 hours in the dark in an environmental chamber at the same temperature as the whole core incubation performed the day prior. To end the incubation, we extracted 5 mL of gas from the vials using a gas-tight syringe and transferred the sample to evacuated 10 mL hungate tubes. The samples were stored underwater up to a maximum of 2 weeks until analysis.

### *Analytical Methods*

We measured ethylene concentrations using a gas chromatograph with a flame ionization detector (GC-FID) using a Porapak N 80/100 packed column. For samples collected from the whole cores, the serum bottles were stored on ice to stabilize the temperature 30 minutes prior to analysis because the solubility of ethylene in water is temperature sensitive. Samples were shaken to equilibrate the ethylene in the headspace and water, and subsequently 5 mL of headspace were extracted using a gas-

tight syringe and immediately run on the GC-FID. For ethylene samples collected from sediment plugs, 5 mL of gas were extracted from the hungate tubes and immediately injected into the GC-FID. Ethylene standards for the whole core and sediment plug incubations were prepared immediately before analysis using 100ppm and 1000ppm ethylene in nitrogen gas standards (AirLiquide). All standards were handled in the same manner as incubation samples. For the whole core incubation standards, a known volume of standard ethylene gas was injected into a serum bottle containing 50 mL of filtered tidal water from each site (0°C). The bottles were immediately shaken and 5 mL of headspace gas extracted for analysis on the GC-FID. For the sediment plug incubation standards, we injected a known volume of ethylene gas prior to analysis into a 40 mL vial containing 5 cm<sup>3</sup> of inert rubber septa. Then 5 mL of headspace gas from the vials were extracted and transferred into evacuated hungate tubes. Finally, 5 mL of gas were extracted from the hungate tubes and injected into the GC-FID for analysis.

Filtered ambient tidal water and porewater-overlying water samples collected for the determination of  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$  concentrations were run on a Lachat Instruments Quik Chem 8000 flow injection analyzer. We used an 861 Advanced Compact Ion Chromatograph with a Metrosep A supp 5 column to measure bromide concentrations for tracking the volume of overlying versus porewater in our mixed samples.

### *Statistical Analysis*

To measure ethylene production in the whole-core incubations, we sacrificed single sediment cores at time points throughout the incubation to measure N fixation

activity over time. We used the slope of linear regressions (4-5 point minimum) to calculate a monthly rate per marsh (see Appendix A-4 for sample regression). By using the slope of the ethylene accumulation rate from the sacrificed cores, and not assuming a time zero, we did not need to separately estimate the contribution of ethylene produced during the “pre-incubation”. In addition to calculating N fixation this way, we also used linear regressions to estimate the rates of nutrient and oxygen uptake and production. Ethylene production was converted to N fixation rates using the theoretical stoichiometric ratio of 3:1 moles of ethylene produced to moles of N<sub>2</sub> fixed. All rates measured using the whole core method were corrected for dilution (which resulted from the gravity flow-through sampling of overlying water) and standardized by the volume of porewater + overlying water mixed together in each core (see Appendices A-1 and A-2 for calculations). Nitrogen fixation rates from the sediment plug incubations were standardized and scaled up to a meter squared area of sediment (see Appendix A-5).

The nine monthly whole core rates generated per site were used to test for significant differences between marshes and among seasons. Seasonal trends were determined by grouping months as follows: Summer (July, August, September); Fall (October, November, December); Spring (April, May, June). We also tested for differences between sites using the sediment plug method, as well as differences between the two sampling methods. Significant differences were evaluated using one-way and two-way ANOVAs for normally distributed data, and Friedman tests (two-way ANOVA equivalent) for non-normally distributed data (Table 2-1). When appropriate, some data sets were square root or log transformed to obtain normality.

In cases with data that were distributed normally but had unequal variances, we used Welch ANOVAs. Relationships between variables were tested using multiple regressions. Standard errors of the linear regressions (i.e. production and uptake rates) were generated using regression analyses on Microsoft Excel (see Appendix A-6 for equations). All other tests were run using JMP Statistical Software (v. 10.0) and SAS Statistical Software (SAS Institute, Inc.).

## RESULTS

### *Nutrients in Ambient Tidal Water*

Mean annual  $\text{NO}_{3+2}^-$  (averaged over the 9 months measured for this study) in marsh-adjacent estuarine waters was  $\sim 3\times$  higher at Little Mussachuck marsh ( $9.0 \pm 3.0 \mu\text{M}$ ) compared to Fox Hill ( $3.3 \pm 1.1 \mu\text{M}$ ), though this difference was not statistically significant (Table 2-1 and Fig. 2-2). Although  $\text{NH}_4^+$  concentrations were also not statistically different between sites, mean annual  $\text{NH}_4^+$  at Little Mussachuck ( $5.3 \pm 1.6 \mu\text{M}$ ) was nearly  $3\times$  higher than at Fox Hill ( $2.0 \pm 0.5 \mu\text{M}$ ). For both  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$ , concentrations were higher at Little Mussachuck during the late summer and fall seasons ( $\sim 4\times$  higher on average), and similarly low at both sites during the spring. In addition, maximum concentrations of  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$  concentrations measured during the annual cycle were greater at Little Mussachuck ( $11.2 \mu\text{M NH}_4^+$ ;  $20 \mu\text{M NO}_{3+2}^-$ ) than at Fox Hill ( $4.5 \mu\text{M NH}_4^+$ ;  $8.2 \mu\text{M NO}_{3+2}^-$ ). However, as part of our site characterization work, we sampled tidal water monthly (in addition to sampling for the

ARA incubations). When we combined all the tidal nutrient data we found that annual average  $\text{NH}_4^+$ , and  $\text{NO}_{3+2}^-$ , and DIN concentrations were significantly higher at Little Mussachuck than Fox Hill (see Ch. 1, Table 1-1; Appendix A-9).

Seasonal changes in  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$  in the estuarine water (collected for the ARA incubations) at both sites were significant (Table 2-1). Previous work documenting annual cycles of surface nutrients in various regions of Narragansett Bay in recent years report similar seasonal trends and concentrations of  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$  in waters near our study sites (Krumholz 2012). Comparing the dissolved inorganic nitrogen data from our study and Krumholz (2012), we assumed the tidal N concentrations at Little Mussachuck marsh on an annual basis are typically 3-4x higher compared to those at Fox Hill.

### ***Sediment Oxygen Demand in Whole cores***

Annual average rates of oxygen uptake from sediment microbial respiration were statistically similar between the two sites ( $59.6 \pm 1.1 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Little Mussachuck and  $45.4 \pm 1.3 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Fox Hill) and rates did not differ among seasons (Table 2-1 and Fig. 2-3). As part of our effort to characterize the sites, we also measured sediment oxygen demand (SOD) in additional, separate cores on a monthly basis. Combined with measurements from this study, annual mean SOD rates were very similar between sites ( $54.5 \pm 3.7 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Little Mussachuck and  $48.0 \pm 3.0 \text{ mmol m}^{-2} \text{ d}^{-1}$  at Fox Hill; Ch. 1, Tables 1-1 and 1-2; Appendix A-10). The lowest rates of SOD occurred during the coldest months in the fall and early spring, and surprisingly in August during a warm incubation. Oxygen uptake and temperature

(8-23°C) tended to vary together over the annual cycle (regression analysis – excluding August,  $r^2 = 0.50$ ,  $F_{1, 14} = 13.8$ ,  $p < 0.01$ ) and SOD ranged from 23.4 to 87.0  $\text{mmol m}^{-2} \text{d}^{-1}$ .

### ***Nutrient Uptake & Production in Whole Cores***

Mean annual  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$  uptake and production in the intact sediment cores did not differ between sites, nor did they vary seasonally (Table 2-1 and Fig. 2-4). Generally, net changes in nutrient concentrations were not dramatic, with the exception of a few incubations in sediments collected from Little Mussachuck. In particular, Little Mussachuck sediments produced relatively high amounts of  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$  in July and October (maximum of  $7.8 \pm 6.0 \text{ mmol m}^{-2} \text{d}^{-1}$ ), which could have been influenced heavily by the amount of porewater mixed into the sample. We also observed relatively high  $\text{NO}_{3+2}^-$  uptake in November ( $-6.6 \pm 3.5 \text{ mmol m}^{-2} \text{d}^{-1}$ ).

### ***Nitrogen Fixation in Whole Cores & Sediment Plugs***

Over the annual cycle, mean N fixation measured in the whole cores was significantly higher at the low N marsh, Fox Hill, than at the high N marsh, Little Mussachuck and rates did not significantly vary on a seasonal basis (Table 2-1 and Fig. 2-5). Similarly, N fixation measured at all depths in the sediment plugs in August, September, November, and June was statistically higher at Fox Hill than Little Mussachuck (Table 2-2 and Fig. 2-6). However we found significant variation among months using the sediment plug method. Comparing N fixation at various depths in the plugs, activity was twice as high in surface sediments (0-5 cm) than deeper sediments (5-10 cm) in

November and June at Fox Hill, but at Little Mussachuck N fixation was similarly low at all depths (Table 2-2). However, we did not find significant differences along the depth profile (0-2 cm, 2-5 cm, and 5-10 cm; Table 2-1). Comparing all of the measured rates during the months when we used both whole cores and sediment plugs, we found that N fixation significantly differed between methods (Table 2-1 and Fig. 2-6). Average N fixation from sediment plugs integrated to a depth of 5 cm at Fox Hill ( $256.0 \pm 59.5 \mu\text{mol m}^{-2} \text{d}^{-1}$ ) and Little Mussachuck ( $199.3 \pm 71.3 \mu\text{mol m}^{-2} \text{d}^{-1}$ ) was over 5-6x higher than N fixation measured in intact sediment cores in the same months ( $53.9 \pm 14.1 \mu\text{mol m}^{-2} \text{d}^{-1}$  at Fox Hill and  $32.1 \pm 14.6 \mu\text{mol m}^{-2} \text{d}^{-1}$  at Little Mussachuck).

## DISCUSSION

### *Effect of Nutrient Enrichment on Nitrogen Fixation*

Using whole cores and sediment plugs, we observed that N fixation was highest at Fox Hill, the marsh with low N enrichment. Annual mean N fixation measured in whole cores was more than 2x higher at Fox Hill than Little Mussachuck, the marsh with high N enrichment (Figs. 2-5 and 2-6). The difference in N fixation between the two sites was more pronounced during the summer and early fall. Though this trend was fairly consistent, we did observe times when N fixation was similar between sites, particularly in the spring (using both incubation methods) and in September (in the sediment plugs only).



Because N fixation is limited by various factors, it is important to consider the results of our study in context of other controls, such as carbon availability and temperature, in addition to DIN concentrations. In both vegetated marsh and seagrass-dominated sediments, researchers have found that heterotrophic N fixation is closely associated with labile sediment C content and plant root exudates, while others have found that N fixation is stimulated by organic carbon amendments (e.g. Hanson 1977; Dicker and Smith 1980a; Hanson 1983; Yoch and Whiting 1986; Talbot et al. 1988; Blaabjerg and Finster 1998; McGlathery et al. 1998). If C availability were primarily limiting N fixation in the marshes we studied, we would expect to see a positive relationship of carbon to heterotrophic N fixation in the sediments. However sediment oxygen demand (SOD), which is an indicator of microbial C availability, was statistically similar between sites over the annual cycle in the whole core incubations. Even in July and May, when SOD was higher at Little Mussachuck, N fixation was lower or similar to Fox Hill rates. Furthermore, to characterize our sampling sites we measured sediment C and N content and along depth profiles (down to 15cm) in cores collected within the sampling area of each site. Percent C and N were higher at Little Mussachuck than Fox Hill, though C:N ratios were similar (see Ch. 1, Table 1-1). Therefore we suggest that exposure to high DIN levels at Little Mussachuck, as evidenced by higher tidal  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations (Fig. 2-2), was likely suppressing N fixation activity, even though C availability was comparable or higher to that at Fox Hill.

Other fertilization studies on salt marsh sediments report varying results in the response of N fixation activity to DIN (ammonium, nitrate, and/or urea) amendments.

Some studies have observed a clear inhibition of N fixation from experimental fertilization. Van Raalte et al. (1974) and Carpenter et al. (1978) reported inhibition of nitrogenase activity by high concentrations of  $\text{NH}_4^+$  in a Cape Cod marsh. In a southern California estuary, Moseman-Valtierra et al. (2010) observed a marked decrease in N fixation rates in vegetated marsh sediments following 17 days of fertilization with  $\text{NH}_4^+/\text{NO}_3^-$  enriched seawater. Although other studies have also documented suppression of N fixation by N fertilization, these trends are often spatially and temporally variable. For example, Dicker and Smith (1980b) found that short-term amendments of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  during assays inhibited N fixation, though this was dependent on the season and the species of nitrogen used. In the rhizosphere of *S. alterniflora* in a South Carolina marsh, long-term fertilization over the course of one year with ammonium-nitrate resulted in N fixation inhibition during some sampling dates, but not all, and in some plots previously suppressed activity resumed at later dates (Bagwell and Lovell 2000). Similarly in our study, though annual N fixation was lower at our high N marsh over the total 9-month period, we observed similar rates between marshes during the springtime in particular (Figs. 2-5 and 2-6). Additionally, though several studies have documented alterations in N fixation activity due to N enrichment, recent work utilizing genomic tools has found evidence that the community composition and abundance of diazotrophs are resilient to changes in N regime (Piceno et al. 1999; Bagwell and Lovell 2000; Moseman 2007).

The relationship of N fixation to plant activity has been noted in several studies investigating the impact of N enrichment. For example, in a *S. alterniflora* dominated marsh in South Carolina, short-term (2-4 hours) amendments of  $\text{NH}_4^+/\text{NO}_3^-$  during

acetylene incubations resulted in spatially dependent N fixation inhibition, with suppression of rates specifically associated with root and rhizome N fixers as opposed to bulk sediments (Yoch and Whiting 1986). In a tropical phosphorus (P) limited marsh, long-term P amendments resulted in enhanced N fixation paired with increased belowground plant biomass, while N amendments resulted in lower rates of N fixation (Černá et al. 2009). In N-limited marshes, the potential for fertilization to enhance plant growth and productivity in nutrient-limited marshes can override the suppression of N fixation by enhancing carbon input from plant roots. For instance, Hanson (1977) observed an increase in N fixation with  $\text{NH}_4^+/\text{NO}_3^-$  fertilization over a 5-month period, while Piceno and Lovell (2000) reported that N fixation was initially stimulated with 2 weeks of fertilization but was not measurably affected with an 8-week fertilization treatment.

Although we found a generally negative relationship of N fixation to tidal  $\text{NH}_4^+$  levels, higher N fixation activity was coupled with seasonally high  $\text{NH}_4^+$  concentrations during the early fall at both sites (Figs. 2-2 and 2-5). Rates peaked at both sites in the early fall, but N fixation was notably much lower at the high N marsh, which could indicate a higher level of suppression by higher DIN in the tidal water. During the fall the peak in N fixation could have been stimulated by warmer temperatures in combination with the end of the growth season and senescence of the plants when root exudation of carbon tends to peak (Welsh 2000). In temperate systems N fixation can be positively coupled to high periods of root exudation of labile C resulting from peak macrophyte primary production and to the decay of roots and rhizomes following the growing season (e.g. Dicker and Smith 1980a; Whiting et

al. 1986; Welsh et al. 1996; McGlathery et al. 1998; Welsh 2000). Although we observed a measurable suppression of N fixation by long-term exposure to high DIN concentrations at Little Mussachuck, seasonal changes in sediment N fixation at both sites seemed to be additionally influenced by temperature and seasonal cycling of plant growth and activity. In contrast, N fixation activity was relatively low during the plant growth season (spring and summer) and also during colder months. Other studies of seasonal N fixation in temperate salt marshes have observed similar trends, with the highest rates in the late summer and early fall and lowest activity during cold months (e.g. Jones 1974; Carpenter et al. 1978; Patriquin and McClung 1978; Dicker and Smith 1980a; Tyler et al. 2003). In salt marsh and seagrass-dominated sediments, studies have reported that seasonal trends in N fixation are closely associated with seasonal shifts in plant productivity, belowground biomass, and root exudation.

### ***Comparison of Incubation Methods***

In salt marsh sediments, researchers have employed a variety of incubation techniques to measure N fixation using the ARA method. These techniques include *in situ* incubations placing gas tight chambers on the marsh surface, collecting cores and incubating small subsamples of sediment in containers (bottle incubations), or incubating intact sediment cores. In addition, incubations are carried out either under non-inundated (typically in air or oxygen-free atmospheres) or inundated conditions (in seawater). We found significant differences between methods in the estimation of N fixation rates in our comparison of two incubation techniques (small sediment plug samples incubated in an oxygen-free atmosphere versus whole cores under flooded

conditions). In three out of four monthly incubations, measured rates were higher in the sediment plugs than the whole cores (Fig 2-6). This is likely the first direct comparison of sediment plugs and whole cores in a salt marsh, so it is unknown if these results are wholly representative especially outside of the temperate region. However, a similar comparison of ARA incubation methods in a seagrass bed supports our results, finding higher N fixation in bottle incubations of sediment “slurries” (small sediment samples mixed into seawater) compared to inundated whole cores (Welsh et al. 1996). A review of N fixation in seagrass sediments by Welsh (2000) discusses the potential for overestimation using slurry techniques due to the release of labile C from plant materials during sample preparation and the potential for underestimation using whole cores due to uneven diffusion and saturation of acetylene throughout vegetated sediments.

A large advantage of bottle incubations of small sediment samples, either as plugs in aerial conditions or as slurries in seawater, is the relatively simple set-up and execution of incubations, which allows for more replication and a larger sample size. In addition, this method is conducive to making measurements at depth by sectioning and subsampling sediment cores. In cores used in this study collected from Fox Hill and Little Mussachuck, we were able to compare rates along a depth profile down to 10cm using sediment plugs, which provided us with vertical N fixation data that we were not able to capture with our whole core method.

The largest drawback to incubating sediment plugs and slurries is the unavoidable disruption of the sediments, which results in altering porewater chemistry and associated microenvironments, disturbing the microbial community, and breaking

up roots and rhizomes in vegetated sediments. While this is also a concern with the whole core method, which cuts the roots and rhizomes during coring, the majority of the sediment structure remains intact and undisturbed. As a result of the high level of disturbance in sediment plugs and slurries particularly, measured biogeochemical processes very likely do not represent ambient rates. In vegetated sediments and particularly in the rhizosphere the disturbance and handling of belowground plant biomass results in the release of significant amounts of labile C (Hansen and Lomstein 1999), which can artificially stimulate microbial activity. It is plausible that the elevated rates we observed using the sediment plug method were due, at least in part, to stimulation of N fixation by higher C availability from breaking up belowground plant biomass. Another inherent weakness in this method is that typically small amounts of sediments are incubated. In this study we incubated sediment samples 5 cm<sup>3</sup> in volume. Although these samples were previously homogenized and subsampled from a larger section, N fixation rates must be scaled up considerably (vertically with depth and horizontally over a specified area), which could contribute to significant error in estimated rates, especially if there exists substantial spatial heterogeneity in the sediments (Dicker and Smith 1980a). The variation in N fixation between replicate cores in our sediment plugs was relatively small however, indicating potentially minimal error due to spatial heterogeneity within and among the cores.

Our motivation in developing a whole core incubation method was to better preserve the integrity of the sediment structure, thereby capturing N fixation activity that is more representative of ambient rates. In addition the “pre-incubation”, in which we saturated the top layer of sediments with acetylene-amended tidal water

prior to sampling, allowed us to avoid a potential “lag period” in our measurement of ethylene production. This lag period, associated with the slow diffusion of acetylene gas into the sediments from overlying water, has been documented in several ARA salt marsh studies (David and Fay 1977; Teal et al. 1979; Dicker and Smith 1980b).

Though these are some considerable advantages of using this method over sediment plugs or slurries, there are several potential sources for error associated with the whole core technique that likely contributed to the large variation we observed among cores in some of our incubations. First, although initial tests using a bromide tracer showed that the “pre-incubation” period allowed for the tracer-amended water to be introduced in the sediment down to 6 cm (Appendix A-7), the tracer likely did not spread throughout the sediment uniformly. Variations in the density and composition of the sediments, which include pockets of air, likely contribute to uneven saturation of sediments. Although we tried to release as many air bubbles from the sediment as possible prior to starting the incubations (via tapping the cores gently), it was impossible to rid the sediments of all air pockets. Other factors could also account for large variation among cores and potentially contribute to underestimating N fixation, such as the potential loss of acetylene while setting up the experiment, loss of ethylene while mixing the porewater into the overlying water. In addition, we were only able to capture activity in the top layer of sediment (~ top 3-6 cm) using this particular whole core incubation technique. Though this captured some of the rhizosphere N fixation, the total N fixation occurring deeper within the sediments was not represented. In addition, not knowing the exact depth to which we

were sampled in the cores likely contributed to potential error in comparing depth-integrated N fixation rates to the sediment plugs and other studies in the literature.

We also documented high variability in the volume of porewater released into the overlying water upon breaking up the top layer of sediment (ranging 0.03 – 0.34 L of porewater that was mixed into 1.0 – 1.6 L of overlying water). The large range in porewater volume could be due to the presence of air pockets and the variable depth in the sediment that was broken up prior to sampling. This could explain the lack of trends we observed in nutrient fluxes in the porewater + overlying water mix. Despite the variation in the volume of porewater released, we were able to capture more N fixation fairly consistently using this sampling method (Appendix A-8) as opposed to sampling the overlying water alone, which is limited by the rate of ethylene diffusing out of the surface sediment and likely captures only surface sediment activity. Averaged over the entire 9 month period, we captured 47% more N fixation by additionally sampling porewater in the top 4 cm sediments at Fox Hill, and 16% more N fixation at Little Mussachuck, compared to sampling the overlying water alone.

Despite the considerable variation among replicate cores using the whole core method, we observed a clear difference in N fixation between the two marshes and conclude that overall trends in N fixation are sufficiently captured using the whole core technique. Although it is likely that this method yields underestimates of absolute rates of N fixation whereas the plug method likely overestimates rates, we suspect that by preserving the sediment structure intact cores likely yield estimates that more closely resemble ambient N fixation. It is important to consider that the absolute difference in N fixation between the methods is sensitive to the depth to



which we captured and extrapolated rates. In this study, we extrapolated N fixation in the sediment plugs to a depth of 5cm to compare the rates captured in the cores (which captured activity to an approximate range of sediment depth of 3-6 cm). The extrapolation to deeper depths for the sediment plugs effectively increases the estimated N fixation rate. However, even if we were to conservatively extrapolate the rates to a depth of 3 cm, mean sediment plug N fixation ( $127.0 \pm 34.3 \mu\text{mol m}^{-2} \text{d}^{-1}$  at Fox Hill and  $96.7 \pm 47.9 \mu\text{mol m}^{-2} \text{d}^{-1}$  at Little Mussachuck) would still be greater than mean rates measured in intact cores during the same months ( $53.9 \pm 14.1 \mu\text{mol m}^{-2} \text{d}^{-1}$  at Fox Hill and  $32.1 \pm 14.6 \mu\text{mol m}^{-2} \text{d}^{-1}$  at Little Mussachuck).

The comparison among incubation methods needs considerably more investigation though, and further refinements to the methodology to address the limitations discussed above. Additionally, both techniques yielded rates that were within the lower range of reported salt marsh N fixation. In a review of salt marsh nitrogen dynamics Hopkinson and Giblin (2008) listed N fixation rates (measured using the acetylene reduction assay) from the literature, resulting in a large range spanning 0 to  $181 \mu\text{mol N fixed m}^{-2} \text{hr}^{-1}$ . Mean annual rates from our study ranged from 0.3 to  $16.5 \mu\text{mol N fixed m}^{-2} \text{hr}^{-1}$  between both incubation methods. Other studies in the nearby region (i.e. mid and northeast U.S. and southeast Canada) ranged from 0.3 to  $150 \mu\text{mol N fixed m}^{-2} \text{hr}^{-1}$  (Table 2-3). Although we measured significantly different rates between the two methods, the difference was very small in comparison to the range reported among various studies.

## *Conclusions*

In coastal systems such as salt marshes, where N fixation is often an important source of N to plant growth, high loading of anthropogenic N has the potential to reduce or alter N fixation. Our study documents a general suppression of N fixation in vegetated marsh sediments due to high N enrichment, though this seemed to be seasonally dependent. At the highly N-enriched marsh, tidal water DIN reached maximum concentration of 28 $\mu$ M, which is low compared to other heavily N-loaded estuarine systems that exceed DIN concentrations of 150 $\mu$ M. The fact that we still observed significantly lower N fixation in our enriched marsh indicates sensitivity of rhizosphere N fixation to N enrichment. The addition of anthropogenic N in salt marshes is not necessarily a straightforward substitute for the function provided by N fixation to marsh plants. The shift from the plant use of N produced by mutualistic diazotrophs to exogenous N has not been well studied, but could potentially be responsible for some of the associated impacts of external N enrichment documented on the marsh plant community (Levine et al. 1998; Boyer and Zedler 1999; Emery et al. 2001; Wigand et al. 2003; Moseman-Valtierra et al. 2010). Food webs may also be impacted, specifically via macrofauna that feed on cyanobacteria and rhizosphere diazotrophs (Moseman-Valtierra et al. 2010). Future studies of N fixation and anthropogenic N enrichment that also incorporate interactions with the plant community and grazers will be necessary to better understand these dynamics.

Our results also indicate that it is important to measure N fixation over time, as activity is affected by seasonal changes in temperature and plant dynamics. For instance, had we conducted our study in the springtime only, we would not have seen

the impact of N enrichment. Long-term studies that measure N fixation multiple times are necessary to capture inherent variations in microbial activity and still be able to observe significant overall trends. The results of our comparison of incubation methods demonstrate that rates of N fixation may depend on the incubation method employed. We recommend that future studies conduct additional incubation comparisons, including whole core methods such as the one employed in this study, which have potential to better represent ambient N fixation rates.

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Table 2-1. Statistics results for nitrogen fixation incubations. Statistics tests used, F ratios and degrees of freedom (test and model error), and significance of tests for differences between sites and among seasons, as well as depth and method comparisons for nitrogen (N) fixation. All variables had replication of n = 9 per site and n = 3 per season, representing 9 monthly measurements, with the exception of N fixation in sediment plugs, which had n = 47 per site n = 3 per month (season). An asterisk on the *p*-value denotes significance (where  $p \leq 0.05$ ). Dashes represent results that are not applicable or available based on the specific test run.

Variable	Test Used	F Ratio (Degrees of Freedom)				p-value			Data	
		Site	Season	Interaction		Site	Season	Interaction	Transformation	
<u>Nitrogen Fixation</u>										
Intact Cores	Two-way ANOVA	4.91 (1, 12)	0.32 (2, 12)	0.50 (2, 12)	-	0.05*	0.74	0.62	-	Square Root
Sediment Plugs	Two-way ANOVA	6.17 (1, 85)	113.30 (3, 85)	1.45 (3, 85)	-	0.02*	<0.0001	0.26	-	
Sediment Plugs - Depth Comparison	One-way ANOVA	-	-	-	0.12 (2, 90)	-	-	-	0.89	
Method Comparison	One-way ANOVA	-	-	-	16.49 (1, 14)	-	-	-	0.001	
<u>Nutrients</u>										
Ambient Tidal NH <sub>4</sub> <sup>+</sup>	Welch ANOVA	2.47 (1)	15.30 (2)	-	-	0.14	0.002*	-	-	Square Root
Ambient Tidal NO <sub>3+2</sub> <sup>-</sup>	Friedman Test	0.82 (1, 14)	13.21 (2, 14)	-	-	0.38	0.0006*	-	-	
NH <sub>4</sub> <sup>+</sup> in Cores	Two-way ANOVA	0.48 (1, 12)	0.69 (2, 12)	0.65 (2, 12)	-	0.50	0.52	0.54	-	Log (Natural)
NO <sub>3+2</sub> <sup>-</sup> in Cores	Friedman Test	0.14 (1, 14)	0.43 (2, 14)	-	-	0.71	0.66	-	-	
Sediment O <sub>2</sub> Demand	Two-way ANOVA	3.29 (1, 12)	2.60 (2, 12)	0.79 (2, 12)	-	0.09	0.12	0.48	-	

Table 2-2. Depth comparison of nitrogen fixation in sediment plugs. Mean nitrogen fixation ( $\pm$  standard error) extrapolated to an area of 1 m<sup>2</sup>, and standardized per cm depth of sediment is shown for each site. Rates were measured in August, September, and November of 2011 and June 2012. Dashes represent measurements that were not made.

	Nitrogen Fixation ( $\mu\text{mol N}_2$ Fixed m <sup>-2</sup> d <sup>-1</sup> )			
	August	September	November	June
Little Mussachuck (High N marsh)				
0 - 2 cm	30.3 $\pm$ 5.6	68.6 $\pm$ 13.0	68.3 $\pm$ 10.4	28.4 $\pm$ 4.5
2 - 5 cm	34.0 $\pm$ 7.2	-	79.1 $\pm$ 9.5	31.0 $\pm$ 5.7
5 - 10 cm	-	65.5 $\pm$ 11.8	37.0 $\pm$ 8.1	19.9 $\pm$ 3.8
Fox Hill (Low N marsh)				
0 - 2 cm	12.7 $\pm$ 1.4	79.5 $\pm$ 11.5	28.8 $\pm$ 8.4	19.6 $\pm$ 2.0
2 - 5 cm	22.4 $\pm$ 4.1	-	37.2 $\pm$ 10.5	19.5 $\pm$ 3.5
5 - 10 cm	-	46.1 $\pm$ 7.8	22.4 $\pm$ 6.3	15.2 $\pm$ 2.5

Table 2-3. Regional literature comparisons of nitrogen (N) fixation rates. Reported N fixation rates ( $\mu\text{mol m}^{-2} \text{d}^{-1}$ ) are included from studies located along the mid and northeast coasts of the U.S. and the south east shorelines of Canada. ARA refers to the acetylene reduction assay, accompanied by the ratio used to convert ethylene production to N fixation, and an asterisk denotes that the ratio used was calibrated using  $^{15}\text{N}$ .

	N Fixation ( $\mu\text{mol m}^{-2} \text{h}^{-1}$ )	Location	Habitat Type	Season	Sediment Depth	Technique
Current Study	0.3 - 16.5	Narragansett Bay, RI	Short <i>S. alterniflora</i>	Annual cycle	4 cm	ARA 3:1
Carpenter et al. (1978)	0 - 150	Sippewisset Marsh, Cape Cod, MA	Short <i>S. alterniflora</i>	Annual cycle	0.25 cm	ARA 3:2*
8 Dicker and Smith (1980b)	38.9 - 205.1	Lewes, DE	<i>S. alterniflora</i>	Annual cycle	20 cm	ARA 3:1
Curriu et al. (1996)	6	Newport River, NC	<i>S. alterniflora</i>	Summer	1 cm	ARA 3:1*
Patriquin and McClung (1978)	92.3	Nova Scotia, Canada	<i>S. alterniflora</i>	May - Sept	17 cm	ARA
Tyler et al. (2003)	49.7 - 149.1	Hog Island, VA	<i>S. alterniflora</i>	Annual cycle	5 cm	ARA 3:2

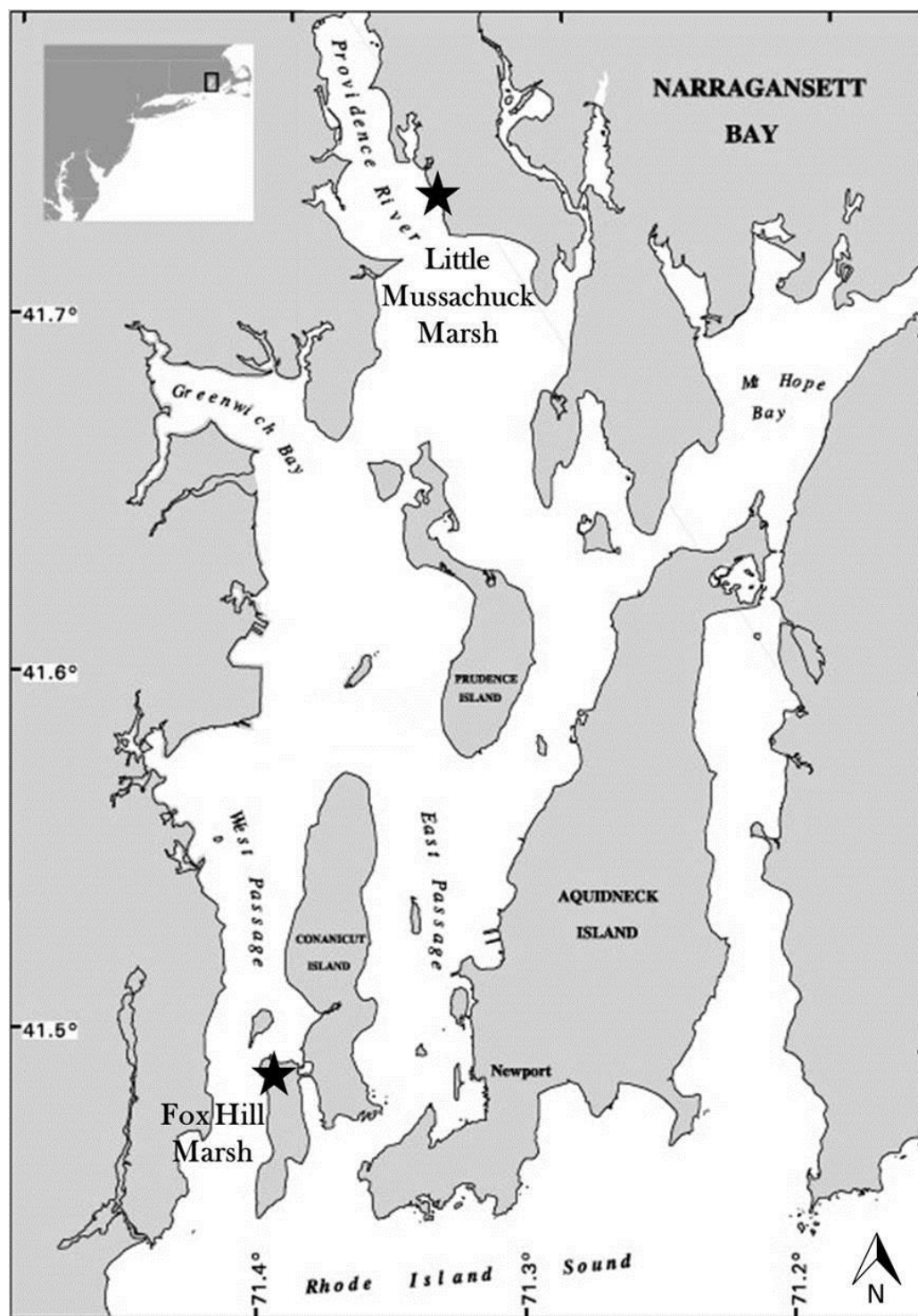


Figure 2-1. Map showing locations of study sites in Narragansett Bay, Rhode Island. Map of Narragansett Bay courtesy of <http://www.gso.uri.edu/phytoplankton/>. Data provided by RIGIS.

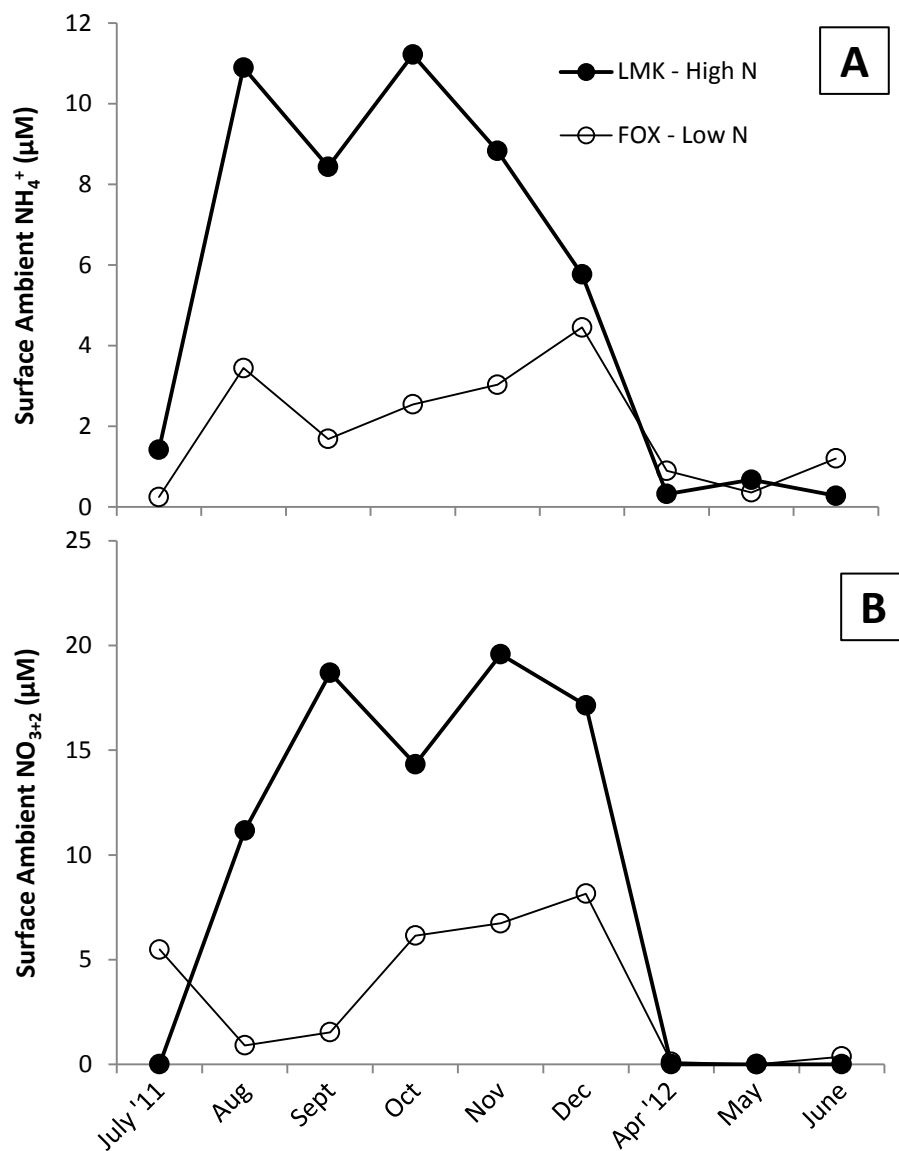


Figure 2-2. Monthly dissolved inorganic nitrogen concentrations in marsh tidal water collected for nitrogen fixation incubations. **(A)** Ambient ammonium concentrations and **(B)** nitrate + nitrite in the surface tidal water measured monthly at two marshes with high and low nitrogen (N) enrichment: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N).

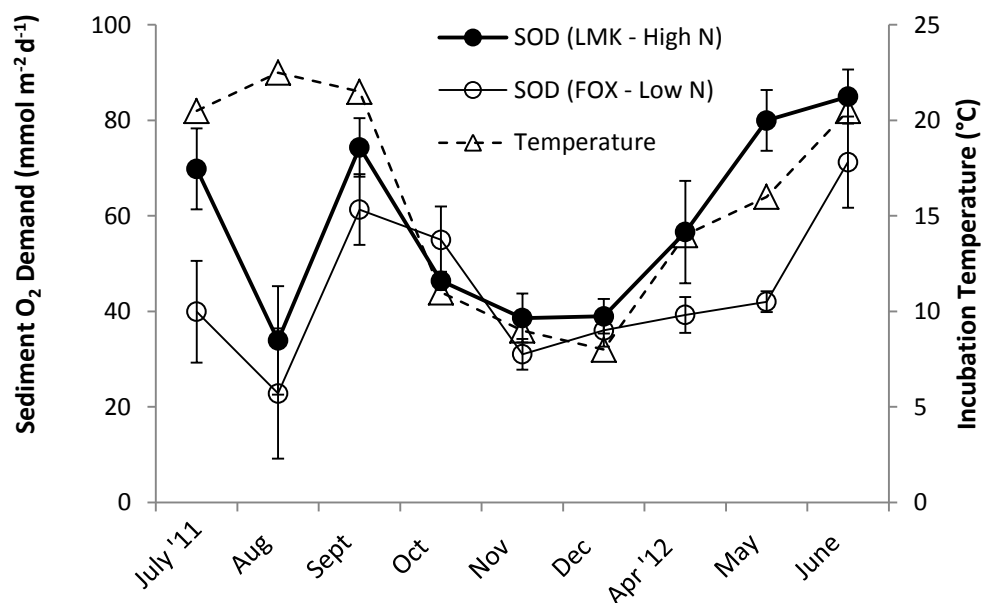


Figure 2-3. Monthly sediment oxygen demand (SOD) in whole core incubations used to measure nitrogen fixation. SOD was measured in two marshes with high and low nitrogen (N) enrichment from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Monthly incubation temperatures were determined from ambient surface soil temperatures measured during the time of sediment core collection. Error bars represent standard error based on linear regressions.

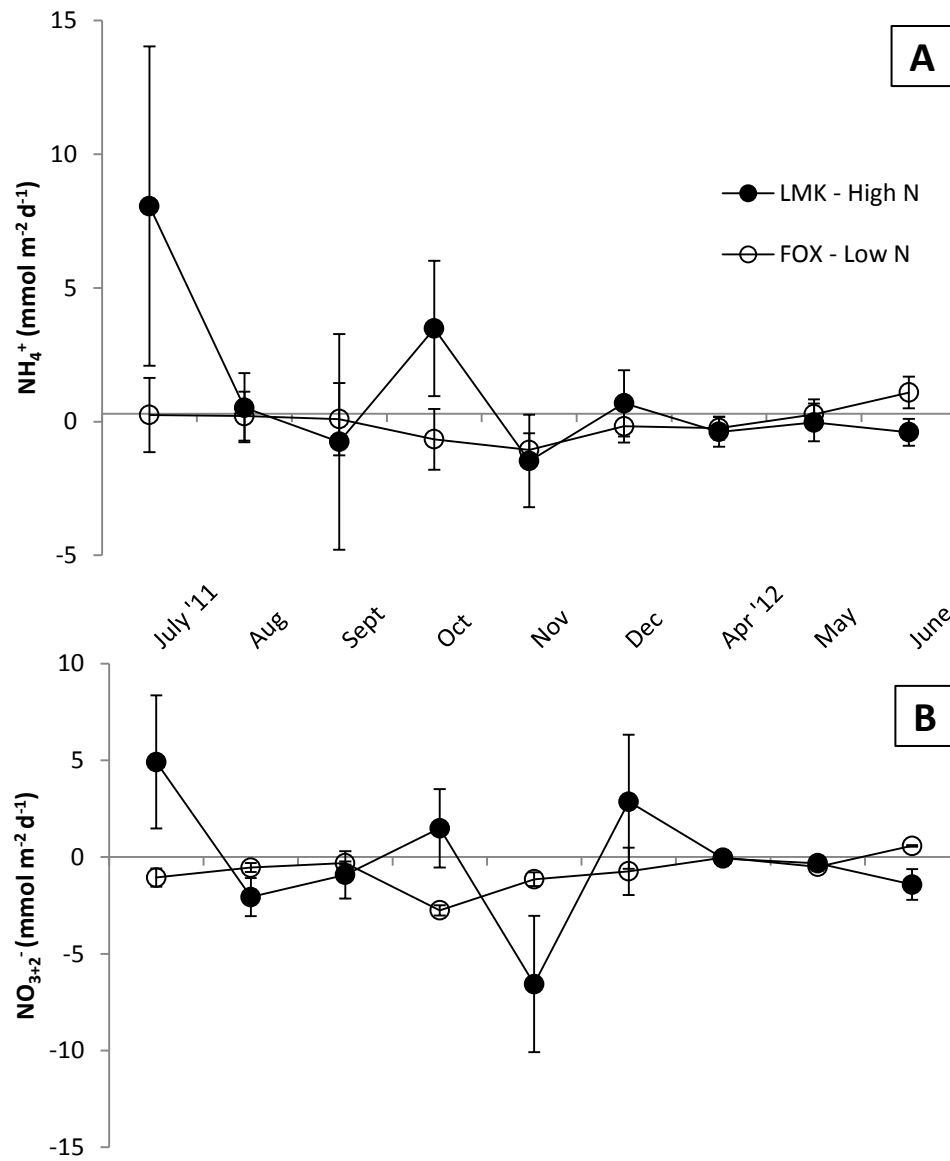


Figure 2-4. Monthly uptake and production of dissolved inorganic nitrogen in whole core incubations used to measure nitrogen fixation. (A) Ammonium and (B) nitrate + nitrite uptake and production in the overlying water and porewater of whole cores measured monthly at two marshes with high and low nitrogen (N) enrichment from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Negative values represent nutrient uptake and positive values represent production. Error bars represent the standard error of the slope calculated from linear regressions.

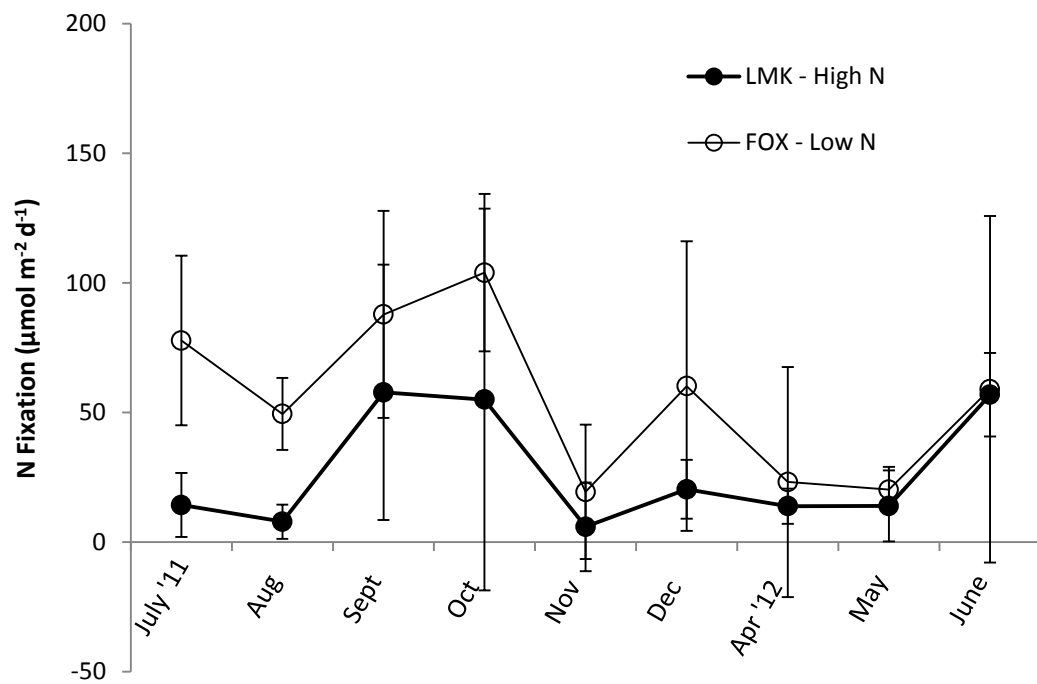


Figure 2-5. Monthly nitrogen fixation measured in whole core incubations. Nitrogen (N) fixation was measured at two marshes with high and low N enrichment from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Error bars represent the standard error of the slope calculated from linear regressions.



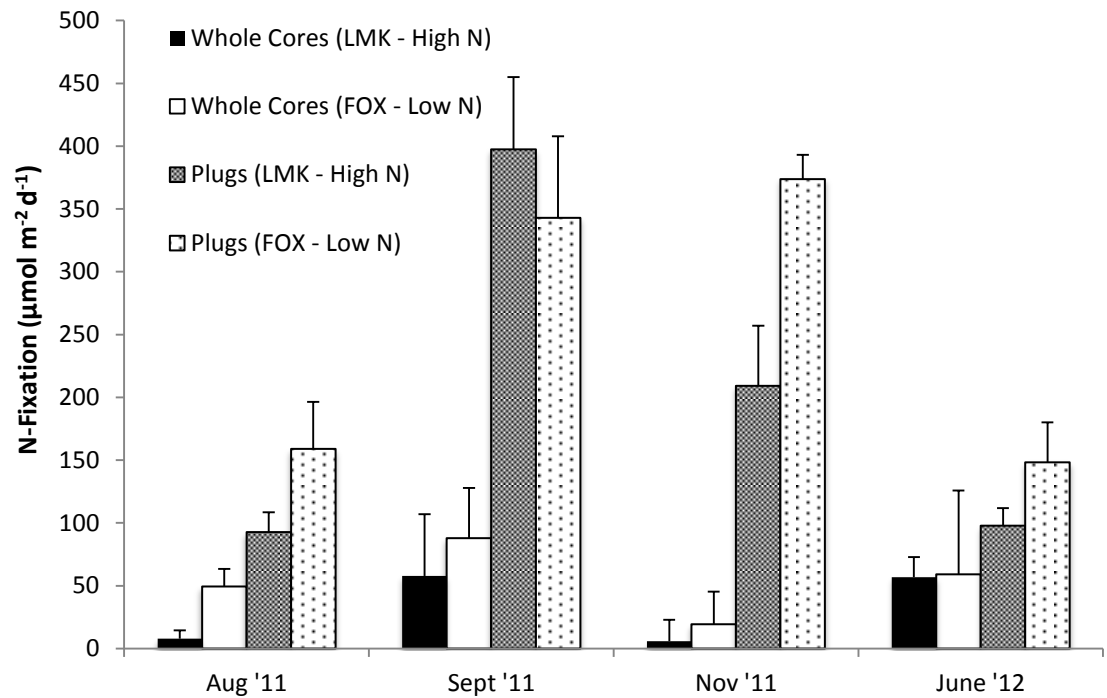


Figure 2-6. Comparison of nitrogen fixation rates measured using the whole core method and sediment plug method. Nitrogen (N) fixation was measured at two marshes with high and low N enrichment from tidal water: Little Mussachuck (LMK - High N) and Fox Hill (FOX - Low N). Rates for the whole cores were measured in the top 3-6 cm of sediment and the plug rates were extrapolated to the top 5 cm of sediment. Error bars represent the standard error of the slope calculated from linear regressions for the whole core method, and standard error calculated from replicate samples for the sediment plug method.

## **CHAPTER 3**

### **PREFACE**

# **THE IMPACT OF NUTRIENT ENRICHMENT ON SALT MARSH NITROGEN FIXATION AND DENITRIFICATION: A TRANSPLANT EXPERIMENT**

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**CHAPTER 3**

**THE IMPACT OF NUTRIENT ENRICHMENT ON SALT MARSH  
NITROGEN FIXATION AND DENITRIFICATION: A TRANSPLANT  
EXPERIMENT**

**ABSTRACT**

With the widespread occurrence of anthropogenic nutrient loading in coastal waters and the implementation of management strategies to reduce nutrient inputs, it is important to understand and characterize how estuaries respond to changes in nutrient regimes. Measuring these responses in salt marshes, which are ecologically valuable systems and efficient transformers of nutrients, is particularly important. Characterizing the microbial processes that serve as sources and sinks for nitrogen by driving nitrogen gas ( $N_2$ ) fluxes in salt marshes is challenging, but is also key to understanding the interaction between marsh nutrient cycling and varying N regimes. We examined this interaction by measuring vegetated sediment nitrogen (N) fixation and denitrification in two salt marshes in Narragansett Bay, Rhode Island, with historically long-term regimes of high and low tidal N concentrations. We also transplanted sediments between the two marshes (i.e. planting sediments from one marsh into the other) and measured N fixation and denitrification after a three-month period to capture short-term responses to changes in tidal N inputs. In addition, we were able to distinguish direct denitrification, coupled nitrification-denitrification, and denitrification capacity using the isotope pairing technique, and separately measure N fixation using the acetylene reduction assay. This enhanced our ability to

comprehensively characterize how changes in N regime affect specific pathways contributing to net N<sub>2</sub> fluxes. Our experiments demonstrated that N enrichment stimulated direct denitrification and suppressed N fixation, while N reductions had the opposite effects. While coupled nitrification-denitrification comprised the majority of ambient denitrification, the impacts to direct denitrification, in particular, generally drove the overall trends among treatments in net N<sub>2</sub> fluxes. We also observed a potential legacy effect on marsh N cycling associated with long-term exposure to a particular N regime, whereby the response of N fixation and denitrification to transplantation resulted in a change in rates towards those of the ambient sediments but not complete convergence. In addition, this legacy effect could be seen in our denitrification capacity measurements. Under conditions where nitrate was not limiting, the capacity for denitrification was highest in the sediments originally from the highly N enriched marsh. This suggests that the microbial community in these sediments was better adapted to efficiently denitrify excess nitrate. The overall findings from this study suggest that external N inputs act as important controls of N fixation and denitrification, driving short-term responses to changes in N regime, as well as shaping microbial activity on longer time scales.

## INTRODUCTION

In recent decades, increases in anthropogenic nitrogen loading and the resulting negative impacts to coastal ecosystems worldwide have garnered much attention within coastal scientific and management communities. The relationship between nutrient loading and salt marshes is of particular interest due to the ecological importance of marshes in coastal systems and their role as nutrient transformers (Valiela and Teal 1979; Nixon 1980; Valiela et al. 2000). As a result, recent work has examined the impact of changes in nutrient loading on marsh structure and function (Gedan et al. 2009) and the ability of marshes to intercept and remove excess anthropogenic nutrients (Teal and Howes 2000; Valiela and Cole 2002; Fisher and Acreman 2004).

Because nitrogen (N) is the limiting nutrient in most temperate coastal systems, most studies have focused specifically on N cycling in the context of nutrient enrichment and processing in salt marshes (Hopkinson and Giblin 2008). While the overall exchange of nutrients between marshes and adjacent tidal waters is fairly well documented (Nixon 1980), nitrogen gas ( $N_2$ ) fluxes between the marsh and the atmosphere are less understood. The balance between nitrogen fixation and denitrification largely controls the net  $N_2$  flux in salt marshes. These processes also serve as important pathways for N inputs and N removal from the ecosystem. Nitrogen fixation can serve as a significant source of new nitrogen to stimulate plant productivity by converting  $N_2$  gas into a biologically available form ( $NH_3$ ), particularly in oligotrophic and young marshes (Tyler et al. 2003; Scott et al. 2007). Denitrification, the microbial mediated process by which nitrate ( $NO_3^-$ ) is transformed

into  $N_2$  gas, effectively removes biologically available N from the marsh. In recent decades many studies have focused on the impacts of N enrichment on salt marsh denitrification in particular, due to the potential for marshes to intercept and remove anthropogenic N from terrestrial sources and adjacent estuarine waters (e.g. Davis et al. 2004; Wigand et al. 2004; Hamersley and Howes 2005; Caffrey et al. 2007; Koop-Jakobsen and Giblin 2010). While this requires further study, our understanding of the impacts of nutrient *reductions* on marsh N cycling is very limited. In many coastal areas management actions to reduce nutrient inputs, such as upgrading septic systems and wastewater treatment facilities, have occurred or will be underway in the near future. As proposed by Fulweiler et al. (2008) from their work in benthic estuarine mesocosms, heterotrophic N fixation in the sediments may increase and dominate the net sediment  $N_2$  flux when an estuarine system transforms from eutrophic to oligotrophic. Therefore, understanding the impact of both N enrichment and N reductions on marsh and estuarine N cycling is becoming increasingly important and relevant.

While the various controls on N fixation such as nutrient concentrations, labile carbon availability, and redox potential have been well studied, the interaction of these factors that regulate N fixation on an ecosystem level complicate our understanding of how N fixation is impacted by environmental change. It is well established that high concentrations of ammonium ( $NH_4^+$ ) and nitrate ( $NO_3^-$ ) can suppress N fixation by limiting the activity of nitrogenase, the enzyme responsible for fixing N (Van Raalte et al. 1974; Carpenter et al. 1978; Dicker and Smith 1980b; Yoch and Whiting 1986). However, studies examining the impact of N enrichment on salt marsh N fixation have

found varying trends, with some studies documenting enhanced or no change in N fixation (e.g. Hanson 1977; Piceno and Lovell 2000) Hanson 1977, Piceno and Lovell 2000), in addition to suppression of N fixation (e.g. Carpenter et al. 1978; Bagwell and Lovell 2000; Moseman-Valtierra et al. 2010). In addition, the impacts of changes in N inputs to salt marshes may also have indirect effects on N fixation. For example, N fertilization has been shown to alter marsh plant productivity, biomass, and community composition (e.g. Wigand et al. 2003), which may have implications for N fixation in the rhizosphere that is closely coupled with plant root dynamics (e.g. Carpenter et al. 1978; Whiting et al. 1986; De Souza and Yoch 1997; Welsh 2000).

Although denitrification in coastal systems is typically limited by  $\text{NO}_3^-$  availability, the impacts of changes in N regime are also not entirely straightforward. Numerous studies have examined the relationship of denitrification to N loading and fertilization, and while some have found higher denitrification activity associated with N enrichment (e.g. Lee et al. 1997; Teal and Howes 2000; Wigand et al. 2004; Hamersley and Howes 2005; Aelion and Engle 2010; Koop-Jakobsen and Giblin 2010), others have reported negative or no clear relationships, or spatially or method-dependent trends (e.g. Nowicki et al. 1999; Davis et al. 2004; Wigand et al. 2004; Tuerk and Aelion 2005; Caffrey et al. 2007; Koop-Jakobsen and Giblin 2010). A majority of these studies employed methods to measure total denitrification, denitrification capacity, or net  $\text{N}_2$  fluxes. Though these approaches provide some important insights, they may not comprehensively capture the denitrification activity specifically associated with changes in external N inputs. Two pathways exist for denitrification; direct denitrification in which external  $\text{NO}_3^-$  is reduced and coupled

nitrification-denitrification that reduces  $\text{NO}_3^-$  internally produced within the sediment. Because direct denitrification is typically limited by external  $\text{NO}_3^-$  inputs, it has the potential to be more sensitive to changes in N loading than coupled denitrification (Koop-Jakobsen and Giblin 2010). While some of this variability among studies may be due to natural variability or the numerous methods employed across studies, it is possible that the effects of N enrichment may be masked by making more generalized measurements of total or net denitrification activity.

While the main objective was to enhance our understanding of the impacts of varying N regimes on salt marsh  $\text{N}_2$  fluxes, we had several specific goals for this study. First, we examined the impact of both N enrichment as well as N reductions on N fixation and denitrification. Our second goal was to evaluate these impacts on short-term and long-term time scales to characterize the ability of these processes to respond and acclimate to varying N regimes. And finally, we sought to separately quantify these impacts on N fixation, direct denitrification, coupled nitrification-denitrification, and denitrification capacity in order to better interpret and understand the implications of our findings. To accomplish these objectives, we designed a transplant experiment in two salt marshes in Narragansett Bay, Rhode Island, that have been exposed to different levels of N enrichment over the last century. We examined the short-term response to changes in N regime by transplanting vegetated sediment cores between the marshes and measured activity three-months following the transplantation. In addition, we also measured activity in sediments that remained in their respective marsh and also used data from previous measurements (Chapters 1 and 2) to compare the long-term impacts of N regime in the two marshes.



## MATERIALS AND METHODS

### *Study Area*

Narragansett Bay, Rhode Island spans an area of 328 km<sup>2</sup> and is a shallow (8.6 meters average depth), phytoplankton-based, and well-mixed estuary with a moderate range in salinity (27-31 psu; (Nixon et al. 1995). The majority of wastewater treatment outflow and riverine inputs are concentrated at the head of the Bay, which is surrounded by a densely-populated watershed (Nixon et al. 2008). This has resulted in a well-established, north-south gradient in nutrient and phytoplankton concentrations, with the highest concentrations in the Providence River Estuary and Upper Bay, and the lowest concentrations near the bay's mouth in the East and West Passages (Oviatt et al. 2002).

We chose study sites for the transplant experiment that were located at opposite ends of the north-south gradient, in the Providence River Estuary and near mouth of the West Passage in Narragansett Bay (Fig. 3-1). The two salt marshes, Little Mussachuck (high N enrichment) and Fox Hill (low N enrichment), have been described in detail in Chapter 1 of this dissertation, including several metrics that we measured to characterize and compare the sites (Ch. 1, Table 1-1). In brief, Little Mussachuck marsh (Barrington, RI; 4.4 hectares) is exposed to relatively high concentrations of dissolved inorganic nitrogen (DIN; ~18-24  $\mu$ M annual average) from estuarine tidal flooding on a daily basis throughout the year, with the exception of the late spring and summer when primary productivity greatly reduces dissolved inorganic (DIN) levels in the surface waters (Krumholz 2012). Surface tidal water

DIN concentrations at Fox Hill marsh (Jamestown, RI; 10.0 hectares), in contrast, are much lower year-round ( $\sim 2 \mu\text{M}$  annual average; (Krumholz 2012). Similar to other New England salt marshes, both Little Mussachuck and Fox Hill are dominated by short-form *Spartina alterniflora* in the low marsh, with tall-form *S. alterniflora* lining the creek banks, and dominated by *Spartina patens* in the high marsh.

### ***Core Collection and Transplantation***

In July 2011 we collected 12 sediment cores from each marsh selected at random within a 0.25 hectare area in the short-form *S. alterniflora* zone. Core collection and re-planting was done within a week period. We collected cores in between plant shoots in order to exclude shoots in the cores. Although the cores were bare on the surface, the sediments contained many roots and rhizomes and therefore were considered to be “vegetated”. The cores (10 cm inner diameter) were extracted by hammering core tubes with sharpened bottoms down to a depth of 20 cm and carefully digging out the core tubes containing intact sediment. The holes in the sediment created by extracting cores were used as spaces for the re-planting of other cores. To account for any residual effects of the transplantation process, 6 of the cores from each marsh were immediately re-planted at random within the original marsh from which they were extracted. We will refer to these as the “control” cores. The remaining cores (6 per marsh) were transported to the opposite marsh where they were re-planted at random that same day. These cores will be referred to as the “transplant” cores. In total we had four treatments, each with 6 cores: Fox Hill Control (Low N marsh), Little Mussachuck Control (High N marsh), Fox Hill Transplant (Low to High

N), and Little Mussachuck Transplant (High to Low N). Half of the cores were used for the N fixation incubation and the other half for the denitrification incubation ( $n = 3$  per treatment, per marsh, per incubation).

If necessary during the re-planting process, sediment was either trimmed off the bottom of the cores or added to the holes so that the surface of the core was flush with the surface of the surrounding sediment. In order to ensure that the re-planted cores would be easily identified and extracted three months later, the outside wall of each sediment core was lined with thin, plastic mesh (2 mm mesh size) that created a permeable barrier between the core and surrounding marsh sediment. The mesh protruded  $\sim 5$  cm above the surface of the sediment. On a bi-weekly basis we checked the mesh to clean off any debris or biofouling, though we never observed any such growth that would have restricted water flow or sunlight penetration over the cores.

The cores remained in the marshes for three months and were collected on 10/6/11 to measure N fixation and 10/12/11 to measure denitrification. The acetylene reduction assay (ARA) was used to measure N fixation in the first incubation. Five days later in a separate incubation we used the isotope pairing technique (IPT) to measure denitrification. Concurrent with extracting sediment cores, we also collected surface tidal water from each site to be used as overlying water for the incubations and to analyze for ambient nutrient concentrations. Immediately following collection, we transported the sediments and tidal water to the University of Rhode Island, Graduate School of Oceanography for processing. The bottoms of the sediment cores were trimmed to obtain a core length of 15 cm and left upright to drain overnight, mimicking low tide. The tidal water collected from both sites was filtered to remove

particles greater than 0.2 microns to eliminate water column N fixation and denitrification activity during the incubations and capture sediment activity only. The cores and tidal water were stored overnight at ambient soil temperatures (21°C for the N fixation incubation and 19°C for the denitrification incubation) in a temperature-controlled environmental chamber and incubated the following day to measure N fixation or denitrification activity.

### ***Core Incubations***

#### *Nitrogen Fixation: Acetylene Reduction Assay*

We used the commonly employed acetylene reduction assay (ARA) method to measure N fixation in the marsh sediment cores (Stewart et al. 1967). The ARA technique is used as a proxy measurement because it is difficult to capture direct changes in N<sub>2</sub> gas from N fixation against the large background of N<sub>2</sub> in the atmosphere. The ARA method instead measures the reduction of acetylene gas to ethylene mediated by nitrogenase, the enzyme responsible for N fixation in diazotrophs. In theory the production of ethylene by nitrogenase, compared to the conversion of N<sub>2</sub> to ammonium, should occur in a stoichiometric theoretical ratio of 3:1 moles (3 moles of ethylene produced for every mole of N<sub>2</sub> fixed). However, calibrations using <sup>15</sup>N<sub>2</sub> tracers of the theoretical ratio performed in coastal marsh sediments have shown that this ratio can vary, reporting ratios of 3:1, 3:2, and 3.6:1 (Carpenter et al. 1978; Teal et al. 1979; DeLaune and Patrick 1990; Currin et al. 1996). Although most measurements of N fixation in salt marshes have been made

using the ARA technique, the majority of published studies forgo the calibration due to the high costs and difficult methods involved. Typically N fixation results are reported as ethylene production, or rates are converted to and reported as N<sub>2</sub> fixation (calculated using a ratio of 3:1 or 3:2 of ethylene produced to N<sub>2</sub> fixed). Despite this inherent weakness, the ARA technique is widely used to measure N fixation in salt marshes and is especially useful for spatial and temporal comparisons within and among studies, or comparing experimental treatments such as those employed in this study.

#### *Denitrification: Isotope Pairing Technique*

To measure denitrification we used the isotope pairing technique (IPT), which has the advantage of distinguishing between coupled nitrification-denitrification and direct denitrification, in addition to comparing ambient activity versus denitrification capacity (Nielsen 1992). Similar to measuring N fixation, various techniques to measure denitrification often employ the use of tracers or proxy measurements due to the difficulty in directly tracking changes in N<sub>2</sub> gas. The IPT method involves adding <sup>15</sup>N-nitrate (<sup>15</sup>N-NO<sub>3</sub><sup>-</sup>) to the system, directly measuring the production of <sup>29</sup>N<sub>2</sub> and <sup>30</sup>N<sub>2</sub> gas over time, and using a series of equations to calculate the production of <sup>28</sup>N<sub>2</sub> gas, representing total ambient denitrification (see Ch. 1, Methods section for a detailed description of equations). In addition, because the amount of <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> tracer is added in abundance such that nitrate in the system is not limiting, the resulting total production of <sup>28</sup>N<sub>2</sub>, <sup>29</sup>N<sub>2</sub>, and <sup>30</sup>N<sub>2</sub> gas represents the capacity for denitrification in the sediments. Finally, by incorporating the ratio of added tracer <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> to naturally

occurring  $^{14}\text{N-NO}_3^-$  in the overlying water, the IPT equations can calculate the proportion of ambient denitrification that occurs as coupled nitrification-denitrification versus ambient direct denitrification. One of the assumptions of the IPT method is that the  $\text{N}_2$  produced originates from the reduction of  $\text{NO}_3^-$ . Although anammox, the reduction of  $\text{NH}_4^+$  to  $\text{N}_2$ , may possibly occur in the marsh sediments, it is likely to be minimal as found in other coastal sediments and marshes (Engström et al. 2005; Koop-Jakobsen and Giblin 2009).

### *Core Incubation Set-up and Sampling Design*

The day following the first field collection in October, we incubated the sediment cores to measure N fixation, and in a second, separate experiment, we measured denitrification by incubating a different set of cores the day following the second field collection. Prior to the incubations, the filtered tidal water from each site was amended with tracer. For the ARA incubations we bubbled a portion of the seawater with acetylene gas for 1 hour to obtain 100% saturation, gently added and mixed the saturated water into a large carboy of un-amended seawater to obtain an approximately 10% acetylene-saturated solution. For the IPT incubations the seawater was amended with  $^{15}\text{N}$ -potassium nitrate ( $\sim 99.9\%$   $^{15}\text{N}$ ) tracer to obtain a concentration of  $160\ \mu\text{M}\ ^{15}\text{N-NO}_3^-$ . In order to ensure that the amended water would have time to saturate the sediments and avoid any initial lag-time in ethylene or  $\text{N}_2$  production at the start of the incubation, we pre-incubated the sediments for  $\sim 3$  hours. First we fitted the core tubes with a gas-tight bottom pieces, and then gently filled up the cores with tracer-amended seawater, which slowly drained through the sediments during the

pre-incubation via a drip-flow port attached to the core bottoms. The physical drainage helped to pull the amended seawater through the sediments (Appendix A-7), mimicking flooding during high tides, and was slow enough so that the cores remained flooded throughout the entire pre-incubation. For the acetylene-amended water, floating foam lids sat on top of the seawater to help slow the escape of the acetylene gas from the surface of the overlying water. We exposed the cores to tidal water that originated from the same marsh from which the cores were collected in October. For both the pre-incubation and regular incubation, cores were kept in the dark at ambient soil temperatures.

To begin the incubation the remaining overlying water was siphoned off and replaced with freshly made tracer-amended, site-specific tidal water. Once filled completely (creating a 43 cm-deep water-column), the cores were capped with gas-tight lids fitted with sampling ports and then placed into a water bath containing a rotating carousel fitted with magnets. The rotating magnets were used to spin floating stir bars anchored in the middle of the water-column in the cores, ensuring mixing of the overlying water throughout the incubation. We incubated the cores for 9 hours to measure N fixation and for 12 hours to measure denitrification. The shorter incubation time for N fixation was used to eliminate some of the error associated with longer ARA incubations (Howarth et al. 1988). At the beginning, middle, and end of the incubation, overlying water samples for analysis of ethylene or N<sub>2</sub> gas and dissolved inorganic nutrients (NH<sub>4</sub><sup>+</sup> and NO<sub>3+2</sub><sup>-</sup>) concentrations were collected mid water-column via a gravity-flow set-up. As water was collected through a sampling

port attached to the core lid, replacement tidal water from a carboy flowed into the core through a different port.

While the shorter incubations ensured that the tracer was not depleted, the shorter time-period was not long enough to allow for porewater production of the gases to come to equilibrium with the overlying water. Therefore we destructively sampled the porewater in the top 3 – 6 cm of each core, sacrificing one core per treatment at the beginning, middle, and end of the incubation. This sampling method provided us with a way to capture a larger portion of rhizosphere N fixation and denitrification as opposed to sampling the overlying water alone. To sample the porewater we used a large metal fork to gently break up the top layer of sediment, mixing the sediment and porewater into the overlying water, and sampling the mixture. A known amount (3 – 5 mL) of bromide tracer added to the overlying water prior to breaking up the sediment was used to determine the volume of porewater mixed into the water-column (Appendix A-1). Duplicate samples (6 mL) were collected before and after breaking up the sediment, and stored at 4°C until analysis for bromide concentrations.

At the beginning of each sampling event, we recorded the water-column oxygen levels by inserting a Hach HQ30 LDO probe into an opening in the core lids. The oxygen concentrations were used to determine sediment oxygen ( $O_2$ ) demand, which indicates sediment carbon availability to the microbial community (see Ch. 1 *Methods* section), as well as to ensure that the water-column did not become hypoxic ( $<4$  mg/L  $O_2$ ) during the incubation. For the ARA incubations, we then collected duplicate samples 50 mL volume for ethylene production, stored in serum bottles with



10 mL of air headspace and fixed with 1 mL of zinc chloride. To analyze N<sub>2</sub> production in the IPT incubations, we collected duplicate water samples 12 mL in volume stored in Labco exetainers and fixed with 0.2 mL zinc chloride. All gas samples were stored underwater at incubation temperatures until analysis. Water samples (60 mL) for analysis of nutrient concentrations were collected last, filtered through 0.45 µM Whatman glass microfiber filters, and stored in acid-washed polyethylene bottles at -15°C until analysis. During porewater sampling, duplicate 6 mL samples were collected for bromide concentrations (before and after breaking up the sediment) and stored at 4°C until analysis.

### *Analytical Methods*

Ethylene concentrations to determine N fixation rates were analyzed on a gas chromatograph with a flame ionization detector (GC-FID) using a Porapak N 80/100 packed column. We stored the serum bottles on ice for 20 minutes prior to analysis in order to stabilize the temperature due to the sensitivity of the solubility of ethylene to temperature. Samples were shaken for 30 seconds immediately prior to analysis, and 5 mL of headspace gas were extracted and injected into the GC-FID. Ethylene standards were prepared immediately before analysis using 100ppm and 1000ppm ethylene in nitrogen gas standards (AirLiquide). All standards were handled in the same manner as incubation samples. A known volume of standard ethylene gas was injected into a serum bottle containing 50 mL of filtered tidal water from each site (0°C). The bottles were immediately shaken and 5 mL of headspace gas extracted for analysis on the GC-FID. For analysis of denitrification rates, <sup>29</sup>N<sub>2</sub> and <sup>30</sup>N<sub>2</sub> dissolved

gas concentrations were analyzed on a quadrupole mass spectrometer without gas equilibrium using a membrane inlet system (Kana et al. 1994). We determined concentrations of ( $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$ ) in seawater collected during the incubations, as well as ambient tidal water, using a Lachat Instruments Quik Chem 8000 flow injection analyzer. Bromide concentrations were determined using an 861 Advanced Compact Ion Chromatograph with a Metrosep A supp 5 column.

### *Statistical Analysis*

Rates of N fixation and denitrification were determined by plotting the concentrations of ethylene or  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  in the porewater-overlying water mix samples over time, and using the slope of a linear regression to determine production rates (Appendix A-2). At the beginning, middle, and end of the incubation, one core per treatment was sacrificed, and therefore each linear regression was determined from 3 points. Nutrient fluxes between the sediment-water interface and oxygen uptake were determined from 6-point regressions of samples collected multiple times from the water-column throughout the incubation. Standard errors of the slope were generated from each linear regression using a regression statistical analysis on Microsoft Excel (see Appendix A-6 for equations). Because we only generated one rate per treatment per site per variable, we were not able to test for differences among treatments. Ethylene production rates were converted to rates of N fixation using the 3:1 stoichiometric ratio from the ARA method, and denitrification rates were determined using the IPT equations. All rates, included nutrient fluxes and sediment oxygen demand, were corrected for dilution that resulted from the gravity flow-

through system for sampling of overlying water, and then standardized by the volume of the water-column in the cores (plus porewater released if applicable; see Appendices A-1 and A-2 for calculations).

## **RESULTS**

### ***Tidal Nutrient Enrichment***

Nutrient concentrations in the surface tidal water collected for the ARA and IPT incubations were ~5.5x higher at Little Mussachuck compared to Fox Hill (Fig. 3-2). In general concentrations were similar between the two different incubations, with the exception of  $\text{NH}_4^+$  at Little Mussachuck which doubled from 7.3  $\mu\text{M}$  for the ARA incubation to 15.5  $\mu\text{M}$  for the IPT incubation. Because we only collected two samples per site per nitrogen species, we did not test for statistical differences. However, additional data to characterize tidal DIN concentrations over an annual cycle (June 2011 to June 2012, excluding winter months) were collected and reported in Chapter 1 of this dissertation (Table 1-1; Appendix A-9). The annual data show that DIN concentrations were consistently and statistically higher at Little Mussachuck compared to Fox Hill, with an annual average 3.5x higher at Little Mussachuck.

### ***Nitrogen Fixation***

Nitrogen fixation was highest in the “Low N Control” (Fox Hill) cores with a rate of 48.8  $\mu\text{mol m}^{-2} \text{d}^{-1}$ , 7x higher than the rate of 6.9  $\mu\text{mol m}^{-2} \text{d}^{-1}$  found in the “High N Control” cores (Little Mussachuck; Fig. 3-3). Nitrogen fixation in the two

Transplant treatments were similar to one another, with rates of  $29.6 \mu\text{mol m}^{-2} \text{d}^{-1}$  in the “Low to High N” and  $26.6 \mu\text{mol m}^{-2} \text{d}^{-1}$  in the “High to Low N” cores, and were in between those of the Control cores.

### ***Denitrification***

Total ambient denitrification was highest in the “High N Control” (Little Mussachuck) cores ( $647.1 \mu\text{mol m}^{-2} \text{d}^{-1}$ ) and was 39% higher than the “Low N Control” (Fox Hill) cores ( $464.6 \mu\text{mol m}^{-2} \text{d}^{-1}$ ; Fig 3-4A). Ambient denitrification in “Low to High N” and “High to Low N” Transplant cores, with rates of 500.2 and  $418.1 \mu\text{mol m}^{-2} \text{d}^{-1}$ , respectively, were also lower than the “High N” cores. Direct denitrification, compared to coupled nitrification-denitrification, made up the smaller proportion of total ambient denitrification, ranging from 9% to 40% (Fig. 3-4A). The highest rates were measured in Little Mussachuck marsh, regardless of whether or not the sediments originally came from the marsh. The “High N Control” cores had the highest rate overall of  $261.8 \mu\text{mol m}^{-2} \text{d}^{-1}$  and also had the largest proportion – 40% – of total ambient denitrification. Direct denitrification in the “Low to High N” treatment was  $179.9 \mu\text{mol m}^{-2} \text{d}^{-1}$ , comprising 36% of ambient denitrification. In contrast, the lowest direct denitrification activity was found in Fox Hill marsh. The “Low N Control” cores had a rate of  $45.2 \mu\text{mol m}^{-2} \text{d}^{-1}$  and made up 10% of ambient denitrification activity. We observed the lowest rate of direct denitrification,  $38.4 \mu\text{mol m}^{-2} \text{d}^{-1}$ , in the “High to Low N” treatment, comprising 9% of ambient denitrification. For coupled nitrification-denitrification, the “Low N Control” cores had the highest rate of  $419.4 \mu\text{mol m}^{-2} \text{d}^{-1}$ , and the “Low to High N” cores had the

lowest rate of  $320.3 \mu\text{mol m}^{-2} \text{d}^{-1}$  (Fig 3-4A). Coupled denitrification was  $385.3 \mu\text{mol m}^{-2} \text{d}^{-1}$  in the “High N Control” cores and  $379.6 \mu\text{mol m}^{-2} \text{d}^{-1}$  in the “High to Low N” cores.

Denitrification capacity was about one order of magnitude higher than ambient denitrification activity across all treatments (Fig. 3-4B). Similar to ambient denitrification, we observed the highest capacity for denitrification ( $6043.8 \mu\text{mol m}^{-2} \text{d}^{-1}$ ) in the “High N Control” cores from Little Mussachuck. The “Low N Control” cores from Fox Hill had the second highest rate ( $4738.7 \mu\text{mol m}^{-2} \text{d}^{-1}$ ). The sediments from the Transplant treatments had the lowest denitrification capacity, with rates of  $4209.4$  and  $4056.5 \mu\text{mol m}^{-2} \text{d}^{-1}$  in the “Low to High N” and High to Low N” cores, respectively.

### ***Sediment Oxygen Demand***

Sediment oxygen demand (SOD) measured in the sediment cores from the ARA incubation ranged from  $58.3$  to  $82.8 \text{ mmol m}^{-2} \text{d}^{-1}$  (Fig. 3-5A). In comparison, SOD measured in the sediment cores from the IPT incubation was generally lower and with a smaller range of  $57.8$  to  $63.2 \text{ mmol m}^{-2} \text{d}^{-1}$  (Fig. 3-5B). The “Low N Control” sediments ranged considerably between the two incubations, accounting for the lowest ( $57.8 \text{ mmol m}^{-2} \text{d}^{-1}$ , IPT incubation) and highest ( $82.8 \text{ mmol m}^{-2} \text{d}^{-1}$ , ARA incubation) rates measured overall.

### ***Nutrient Fluxes***

Ammonium fluxes in the ARA incubations showed both  $\text{NH}_4^+$  uptake and production in the sediments, with approximate uptake rates of  $-1.5 \text{ mmol m}^{-2} \text{ d}^{-1}$  in the “Low N Control” and “Low to High N Transplant” cores, and production rates of  $0.5 \text{ mmol m}^{-2} \text{ d}^{-1}$  in the “High N Control” and “High to Low N Transplant” cores (Fig. 3-6A). All cores exhibited uptake of  $\text{NO}_{3+2}^-$  in the ARA incubations, ranging from  $-0.9 \text{ mmol m}^{-2} \text{ d}^{-1}$  in “High N Control” cores to  $-0.3 \text{ mmol m}^{-2} \text{ d}^{-1}$  in the “High to Low N Transplant” cores (Fig. 3-6B). In the IPT incubations, the patterns in  $\text{NH}_4^+$  fluxes across treatments were quite different from those observed in the ARA incubations, though the general range in rates were similar. Both the “Low N” and “High N” Control cores had  $\text{NH}_4^+$  uptake around  $-0.4 \text{ mmol m}^{-2} \text{ d}^{-1}$ , the “Low to High N” cores had production of  $0.9 \text{ mmol m}^{-2} \text{ d}^{-1}$ , and there was almost no change in  $\text{NH}_4^+$  in the “High to Low N” sediments (Fig. 3-7A). Similarly the “High to Low N” cores did not show much change in  $\text{NO}_{3+2}^-$  during the IPT incubation (Fig. 3-7B). The other cores showed very high rates of  $\text{NO}_{3+2}^-$  uptake however, ranging from  $-19.0 \text{ mmol m}^{-2} \text{ d}^{-1}$  in the “High N Control” cores to  $-28.2 \text{ mmol m}^{-2} \text{ d}^{-1}$  in the “Low to High N” cores.

## **DISCUSSION**

### ***Nitrogen Fixation***

In examining the impact of N enrichment on salt marsh N cycling, we found evidence of N fixation suppression prompted by short-term (three months) and long-term (more than a century) exposure to high DIN levels. The Control cores, which

were re-planted back into the marshes from which they were originally extracted, had the most dramatic differences in N fixation. The Control cores from Fox Hill, the “Low N” marsh, fixed N at a rate 7x higher than the Control cores from Little Mussachuck, the “High N” marsh (Fig 3-3). In measurements made for another study at the same marshes, similar trends in N fixation were observed over an annual cycle with significantly higher activity at Fox Hill (see Ch. 2, Figs. 2-5 and 2-6). In this study we also observed alterations to N fixation in the Transplant treatments, with a change in rates towards those of the ambient sediments but not complete convergence over the brief 3-month duration of the transplant experiment

A variety of factors can regulate N fixation rates such as carbon availability, temperature, light, oxygen, salinity, and grazing (Vitousek et al. 2002). In addition, the suppression of N fixation by high concentrations of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  ( $>7 \mu\text{M}$  DIN) has been observed in many estuarine systems including seagrass beds, estuarine sediments, and salt marshes (Howarth et al. 1988). Heterotrophic N fixation, in particular, is often limited by carbon (C) availability and many studies in vegetated sediments of seagrass and salt marsh systems have found positive relationships between rhizosphere N fixation and naturally occurring labile sediment C content and plant root exudates, as well as stimulation by organic C amendments (e.g. Hanson 1977; Dicker and Smith 1980a; Hanson 1983; Yoch and Whiting 1986; Talbot et al. 1988; Blaabjerg and Finster 1998; McGlathery et al. 1998). We examined several metrics of C availability and limitation in the sediments from both marshes, including carbon content (% C) and C:N as part of our site characterization measurements (see Ch. 1, Table 1-1), as well as measuring sediment oxygen demand (SOD; an indicator

of microbial C availability) over an annual cycle on a bi-monthly basis (see Ch. 1, Fig. 1-4; Ch. 2, Fig. 2-3; Appendix A-10) and in the cores for this experiment (Fig. 3-5). If C availability were primarily driving N fixation, we would expect to see a positive relationship, with significantly lower C availability in Fox Hill sediments compared to Little Mussachuck; however, we found no evidence to support this. Carbon content was higher at Little Mussachuck, C:N and SOD measured over an annual cycle were statistically similar between sites, and we found no relationship in this experiment between SOD and N fixation among the four treatments.

Alternatively, it seems likely that N fixation was suppressed by exposure to high levels of DIN in sediments collected from Little Mussachuck following re-planting. The concentration of DIN in the tidal waters used for the ARA incubation was over 7x higher for Little Mussachuck compared to Fox Hill (23.2  $\mu\text{M}$  and 3.2  $\mu\text{M}$ , respectively). In addition, ambient porewater concentrations and annual surface tidal DIN were significantly higher at Little Mussachuck (see Ch. 1, Table 1). Furthermore, the cores transplanted from Fox Hill into Little Mussachuck (“Low to High N” treatment) exhibited N fixation rates that were considerably lower than the Fox Hill Control cores, suggesting suppression of N fixation by N enrichment at Little Mussachuck. Additional studies in salt marsh sediments have also documented limited N fixation activity associated with N fertilization. Notably, significant inhibition of nitrogenase activity by high concentrations of  $\text{NH}_4^+$  was first observed by Van Raalte et al. (1974) and Carpenter et al. (1978) in sediments from a Cape Cod marsh. In a southern California marsh following 17 days of fertilization with  $\text{NH}_4^+/\text{NO}_3^-$  enriched seawater, Moseman-Valtierra et al. (2010) observed a significant decrease in vegetated



sediment N fixation. Additional studies have reported spatially and temporally dependent inhibition of N fixation in marshes. In a long-term fertilization experiment over the course of one year in a South Carolina marsh, Bagwell and Lovell (2000) documented suppressed N fixation activity in the rhizosphere of *S. alterniflora* on some sampling dates but not all. Dicker and Smith (1980b) found that the inhibition of N fixation by short-term additions of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  in a Delaware marsh was dependent on the season and the species of N used.

Although we observed a marked decrease in N fixation in the “Low to High N Transplant” cores compared to “Low N Control” cores, this decrease did not reach the low levels of N fixation seen in the “High N Control” cores (Fig. 3-3). One explanation for the partial change could be that following transplantation into Little Mussachuck, the fertilization stimulated plant activity and hence root exudation, which in turn could have stimulated N fixation. As documented in other marsh studies, this potential increase in C availability could have partially overridden the suppression of N fixation by high DIN levels. For example, in a South Carolina marsh Piceno et al. (1999) reported stimulation of N fixation after 2 weeks of fertilization, followed by no measurable effects after 8 weeks. Fertilization was accompanied by a notable increase in aboveground plant biomass. In a 5-month fertilization study, Hanson (1977) also observed the stimulation of plant growth and productivity accompanying enhanced N fixation activity in a Georgia salt marsh. Although our cores did not originally have plant shoots in them when transplanted, by the end of 3 months there were live roots and rhizomes that had grown through the sediments and small shoots coming up through the surface sediment. Therefore it is possible that

plant activity could have been affected by the N enrichment and in turn affected rhizosphere N fixation.

Another possible explanation for partial, but not complete, decrease in N fixation in the “Low to High N Transplant” sediments could be associated with robustness of the diazotrophic community. The cores transplanted into Little Mussachuck originally came from Fox Hill where DIN levels are low year-round and N fixation is typically high (Ch. 2, Figs. 2-2 and 2-5). This indicates a possible legacy effect of N enrichment, in which the diazotroph community at Fox Hill was potentially better adapted to fix N compared to diazotrophs at Little Mussachuck, which have experienced long-term exposure to high DIN levels. Recent genomic work examining the effects of fertilization on salt marsh diazotrophs have found that the community composition and abundances are resilient to changes in N regime, even when N fixation activity was affected (Piceno et al. 1999; Bagwell and Lovell 2000; Moseman-Valtierra et al. 2010). Therefore it is possible that although N fixation was likely suppressed in “Low to High N Transplant” cores due to N enrichment, a higher abundance of diazotrophs or a community composition with more competitive species in the Fox Hill sediments may have counteracted the overall suppression of N fixation. Also, a longer time-period for the transplantation – for example one year – may also provide the sediment microbial community more time to acclimate and respond to the change in environment.

Similar to the findings discussed above, the partial but not complete alteration of N fixation in the “High to Low N Transplant” sediments also indicates that the effects of changing long-term N regimes are not entirely linear. It should be noted that

the high error associated with the N fixation rate for the “High to Low N Transplant” treatment is due to very different activity in the cores that were sacrificed in the middle and end of the incubation. One core exhibited low activity similar to N fixation found in the “High N Control” treatment, and the other exhibited high activity similar to the “Low N Control” treatment. The difference in cores may indicate variable responses to the decrease in N enrichment or may be due to spatial variability in the sediments. With the small number of replicate cores – a limitation of this study – we are unable to definitely attribute the high variation in this treatment to a specific mechanism. Other N fixation studies in salt marshes have not examined the effect of *decreasing* fertilization on long-term N enriched sediments, and more investigation is needed to better understand the impact of nutrient reductions on marsh N fixation.

We did not observe any clear relationships between  $\text{NH}_4^+$  and  $\text{NO}_{3+2}^-$  fluxes to N fixation in the cores (Fig. 3-6). The lack of a trend among treatments in  $\text{NH}_4^+$  is likely due to the concurrence of multiple processes that reduce and produce  $\text{NH}_4^+$  such as remineralization, plant and microbial uptake, nitrification and dissimilatory nitrate reduction to ammonium (DNRA). The majority of  $\text{NO}_{3+2}^-$  uptake was likely driven by sediment denitrification and in part by other nitrate reduction processes found to occur in marsh sediments, such as DNRA, which we discuss in more detail in the next section of this discussion.

### ***Denitrification***

Overall ambient denitrification was greatest in the Control cores from Little Mussachuck compared to all other treatments (30-55% higher), with enhanced direct

denitrification responsible for the higher activity. Direct denitrification was 6x greater in the Control cores from Little Mussachuck than those from Fox Hill, whereas coupled nitrification-denitrification was only fractionally higher in Fox Hill. Because direct denitrification is limited by the availability of  $\text{NO}_{3+2}^-$  from the overlying water, the higher levels of tidal  $\text{NO}_{3+2}^-$  at Little Mussachuck likely drove the enhanced direct denitrification activity we observed in the “High N Control” cores. In another recent study to measure ambient denitrification in the same marshes over an annual cycle, similar patterns were observed, with comparatively greater denitrification at Little Mussachuck due to greater rates of direct denitrification (see Ch. 2, Figs. 1-2 and 1-3). In addition, direct denitrification at both marshes was strongly linked to tidal  $\text{NO}_{3+2}^-$  levels throughout the year. Another study by Koop-Jakobsen and Giblin (2010) also reported that *in situ* direct denitrification was significantly stimulated by long-term fertilization in vegetated marsh sediments in Plum Island Sound, MA. Compared to a reference marsh, tidal  $\text{NO}_{3+2}^-$  levels were >10x greater and direct denitrification was 20x higher in the fertilized marsh sediments, with direct denitrification comprising 94% of total ambient activity during high tide. Another study in the creek sediments and vegetated platforms of a highly N enriched marsh in the Venice Lagoon, Italy, found that direct denitrification generally dominated over coupled-denitrification, particularly in the fall when tidal  $\text{NO}_{3+2}^-$  levels peaked (Eriksson et al. 2003).

In the two marshes we studied in Narragansett Bay, it is important to note the substantial contribution of coupled denitrification to overall ambient denitrification activity. Often coupled nitrification-denitrification is typically favored in salt marshes and coastal vegetated sediments due to root oxidation and high labile C content that

fuels nitrification (Risgaard-Petersen and Jensen 1997; Nowicki et al. 1999; Hamersley and Howes 2003; Hamersley and Howes 2005; Koop-Jakobsen and Giblin 2010). Similar rates of coupled denitrification and SOD, with no significant differences between Fox Hill and Little Mussachuck, were also observed in measurements made over an annual cycle, indicating similar C availability to the microbial community (see Ch. 1, Table 1-2, Figs. 1-2 and 1-4; Appendix A-10). In this study, coupled denitrification and SOD rates were relatively similar among all four treatments.

Although coupled nitrification-denitrification is important in the marshes we studied, N regime greatly stimulated or limited direct denitrification. This is further evidenced by the trends we observed in the transplanted sediments. Ambient denitrification decreased in the cores transplanted from Little Mussachuck into Fox Hill, specifically due to  $\text{NO}_{3+2}^-$  limitation of direct denitrification (Fig. 3-4A). In contrast, the exposure to elevated levels of  $\text{NO}_{3+2}^-$  in cores transplanted from Fox Hill into Little Mussachuck resulted in enhanced direct denitrification, which increased overall ambient denitrification. Many other marsh fertilization studies have also documented increases in denitrification activity due to N enrichment (e.g. Howes et al. 1996; Hamersley and Howes 2005; Aelion and Engle 2010; Koop-Jakobsen and Giblin 2010). In comparison to these studies, the difference in external  $\text{NO}_{3+2}^-$  between the two Narragansett Bay marshes in our experiment was not relatively large (1.7  $\mu\text{M}$  versus 7.6  $\mu\text{M}$   $\text{NO}_{3+2}^-$  in the tidal waters of Fox Hill and Little Mussachuck, respectively). The changes in denitrification we observed in the transplant cores indicate that relatively modest changes in external  $\text{NO}_{3+2}^-$  can significantly alter

denitrification activity. Not all studies examining salt marsh denitrification, however, have found positive relationships to N enrichment, including some conducted in Narragansett Bay (e.g. Davis et al. 2004; Tuerk and Aelion 2005; Caffrey et al. 2007).

A particularly interesting finding was that the enhancement of direct denitrification in the “Low to High N Transplant” cores did not reach the level of activity seen in the “High N Control” cores, indicating that the transplanted cores had a comparatively lower potential for denitrification, even following three-months of exposure to high  $\text{NO}_{3+2}^-$  levels (Fig. 3-4A). The measured rates of denitrification capacity, which represented denitrification of ambient  $\text{NO}_{3+2}^-$  plus the  $^{15}\text{N}$ - $\text{NO}_{3+2}^-$  tracer (added to achieve a concentration of 160  $\mu\text{M}$ ), also demonstrated limited capacity in the cores transplanted into Little Mussachuck. Interestingly, although total denitrification across all treatments was an order of magnitude higher than ambient rates (demonstrating that ambient denitrification was ubiquitously nitrate-limited), capacity was highest in the “High N Control” cores. Comparatively higher denitrification capacity was also observed at Little Mussachuck in separate measurements made over an annual cycle (see Ch. 1, Table 1-2, Fig. 1-3). Together these results demonstrate that there likely exists a legacy effect of long-term N enrichment, enhancing the capacity to reduce high concentrations of  $\text{NO}_{3+2}^-$  by salt marsh denitrifiers. Other studies have reported similar findings of long-term N enrichment impacts on salt marshes. For example, in Plum Island Sound, MA, Koop-Jakobsen and Giblin (2010) found that denitrification capacity measured using the IPT method was an order of magnitude higher than ambient denitrification rates. They also found greater capacity in the creek bank sediments of a fertilized marsh compared

to a reference marsh. Contrary to our work, however, they did not see any difference between marshes in denitrification capacity in sediments on the vegetated marsh platform. Another study in Narragansett Bay marshes using denitrification enzyme assays (DEA) found a positive relationship between denitrification potential and modeled N loading in high marsh sediments (Wigand et al. 2004). No relationship, however, was observed in the sediments from the low marsh. Two other studies in a Cape Cod marsh reported that the percent of N intercepted and removed under long-term fertilization increased from 60-80% in the 1970's to 93% in the 2000's, likely representing an enhancement of N cycling and removal by prolonged exposure to N enrichment (Valiela et al. 1973; Brin et al. 2010).

Because the *total* denitrification measured in the cores was mainly dominated by the direct reduction of  $\text{NO}_{3+2}^-$  (both ambient and the added tracer) in the overlying water, we expected to see a relationship between denitrification capacity and  $\text{NO}_{3+2}^-$  fluxes across treatments in the cores (Figs. 3-4 and 3-7). We did not find any clear relationship in this study or in similar measurements made in another study over an annual cycle in the same marshes (see Ch. 1, Figs. 1-3 and 1-5). The lack of a relationship may indicate that other pathways of nitrate reduction and production are important in these marshes. DNRA in particular is often an important nitrate reduction pathway in aquatic systems with labile carbon-rich sediments and under nitrate-limiting conditions (Burgin and Hamilton 2007). Significant DNRA, comparable to denitrification rates, were reported in fertilized and unfertilized marshes in Plum Island Sound, MA (Koop-Jakobsen and Giblin 2010). Because the  $\text{NO}_{3+2}^-$  uptake rates that we observed in this study were 3-7x greater than measured

denitrification rates, we suspect that DNRA was important in the Narragansett Bay marshes. Uptake by plant roots and other microbes could also significantly contribute to  $\text{NO}_{3+2}^-$  fluxes. Though anammox, which directly reduces  $\text{NH}_4^+$  to  $\text{N}_2$ , could have contributed to our measured  $\text{N}_2$  production it is likely that this was minimal, as anammox activity has been reported to be minimal in New England marshes (Koop-Jakobsen and Giblin 2010). In the “High to Low N Transplant” cores we observed almost no net change in  $\text{NO}_{3+2}^-$ , even though total denitrification was comparable to the other treatments. It is possible that high nitrification activity could have counteracted nitrate reduction and uptake.

### ***Net $\text{N}_2$ Flux and N Removal***

With measured ambient denitrification rates that were one or two magnitudes higher than N fixation, net  $\text{N}_2$  removal from the marsh sediment dominated  $\text{N}_2$  fluxes across all treatments (Figs. 3-3 and 3-4). Ambient denitrification was 10x higher than N fixation in the Fox Hill Control cores and 16-17x higher in the Transplant cores. The difference was most extreme in the N enriched Little Mussachuck Control cores, in which denitrification was 93x higher than N fixation. To better understand if these trends were typical, as well as examining them averaged over an annual cycle (excluding winter) we compared ambient N fixation and denitrification measured at the same marshes from previous studies (Chapters 1 and 2). At Fox Hill ambient annual denitrification was 7x higher than N fixation, and was 23x higher at Little Mussachuck. In pristine marshes, low inputs of N (internal and external) are generally balanced by removal, uptake, and burial (Teal and Howes 2000). Although



denitrification greatly dominated the exchange of  $N_2$  gas on the vegetated marsh platform at Fox Hill, the input of new nitrogen by N fixation to the marsh likely plays a non-trivial role in the nutrient budget of the ecosystem (Teal and Howes 2000).

The shift to a near total dominance by denitrification in overall net  $N_2$  fluxes in the Little Mussachuck sediments (in Control cores and those transplanted into Little Mussachuck, as well as the annual data from previous work) demonstrated that with increased anthropogenic loading, the microbial community responds relatively rapidly to compensate for the change in N inputs. In addition, the response of decreased denitrification and a partial increase in N fixation to the reduction in tidal DIN (seen in the “High to Low N Transplant” cores) showed that nutrient reductions in eutrophic estuaries might have an impact on salt marsh N cycling. In Narragansett Bay for example, mandated nutrient reductions via upgrades in wastewater treatment facilities and septic systems to tertiary treatment are currently being implemented, effectively removing a significant portion of DIN from wastewater effluent. The total reduction of N to the Bay with treatment facility upgrades is estimated to be 30-35% (Krumholz 2012). With the subsequent decline in DIN, it is possible that the contribution of marsh N fixation to net  $N_2$  flux in Narragansett Bay marshes will become increasingly important.

Using tidal inundation data collected as part of our site characterization work (see Ch. 1, Table 1-1; Appendix A-11), we calculated the average percent N removed during high tides via net  $N_2$  fluxes (N fixation balanced by total ambient denitrification) at both marshes in the Control cores. Using ambient surface tidal DIN concentrations from the ARA and IPT incubations and tidal flooding data (Ch. 1,

Table 1-1), we estimated that one m<sup>2</sup> of low marsh is loaded with 709 and 3742  $\mu\text{mol}$  of DIN during an average high tide (duration of 3 hours) at Fox Hill and Little Mussachuck marshes, respectively. While flooded during a high tide, we calculated that 46.9 and 44.2  $\mu\text{mol}$  of DIN m<sup>-2</sup> is removed from the low marsh via the net N<sub>2</sub> flux at Fox Hill (Control and High to Low N Transplant, respectively), whereas 79.5 and 58.4  $\mu\text{mol}$  of DIN m<sup>-2</sup> is removed at Little Mussachuck (Control and Low to High N Transplant, respectively). Although the net N<sub>2</sub> flux is greater at Little Mussachuck in both Control and Transplant cores, the magnitude of difference in DIN loadings only allowed for relatively low % N removal from the water column (2.1% and 1.6%, respectively). Percent N removal was comparatively higher in Fox Hill Control and Transplant cores (6.6% and 6.2%, respectively). These estimates fall within the lower end of the range of % N removal we calculated over an annual cycle in the same marshes (Ch. 1, Table 1-3).

Other studies examining system-wide nutrient exchanges in New England marshes have found that total N removal (including denitrification, plant uptake and burial) is high in fertilized marshes, ranging from 50-93% (Valiela et al. 1973; Drake et al. 2009; Brin et al. 2010). While the increase in denitrification can help to compensate for removing high N inputs in enriched marshes, our findings suggest that denitrification in the marsh platform contribute modestly overall N removal. In contrast, other areas of the marsh that are exposed to tidal waters for longer periods of time, such as the creek bank and bottom sediments, which have reportedly greater rates of denitrification, likely play a greater role in water column N removal (Kaplan et al. 1979; Koop-Jakobsen and Giblin 2010). Our findings also suggest that although

N enrichment may stimulate denitrification in the low marsh, the total proportion of tidal N removed (via denitrification) per unit area may not necessarily increase as well. In highly enriched marshes with large N loads, other factors may play a more important role in enhancing total N removal at the marsh ecosystem level, such as plant uptake, burial rates, and hydrological dynamics.

### *Conclusions*

Our findings demonstrated that external N inputs were important drivers of sediment N fixation and denitrification in the salt marshes we studied, and that microbial activity was able to respond rapidly to changes in tidal N regime. However, it was also apparent that the long-term exposure to a particular N regime had an influence, or legacy effect, on the microbial response, either in partially hampering or enhancing activity. Moreover, the effects of tidal N regime on direct denitrification in particular dominated the overall impact to net N<sub>2</sub> flux, as coupled nitrification-denitrification was similar across all treatments.

As a consequence of increased anthropogenic N loading to coastal systems there has been much interest in understanding the ecological impacts to salt marshes in addition to the potential for marshes to remove some of the excess N. Though the overall contribution to removal of tidal N inputs was relatively small in the marsh platform, our results showed that direct denitrification, and hence N removal, was enhanced by N enrichment on both short and long-term time scales. It is possible that denitrification could also be stimulated in other areas of the enriched marsh (e.g., tidal creek sediments, mudflats), which could have greater impacts on overall N removal.

In contrast, with the increasing occurrence of mandated reductions in nutrient loading to coastal waters, it is equally important to understand how decreases in N inputs will impact salt marshes. Our work demonstrated that overall denitrification decreased likely in response to lower tidal N inputs. This was due to tidal  $\text{NO}_3^-$  limitation on direct denitrification in particular. Substantial coupled nitrification-denitrification, however, remained high, both in the short and long-term. Nitrogen fixation, although much less important than denitrification in the overall balance of  $\text{N}_2$  fluxes between the marsh platform and tidal waters, was also likely affected by the difference in N regime. Clearly, exposure in the long-term to low N inputs resulted in higher rates of N fixation. The effect of reducing N enrichment on N fixation, however, was less clear, due to high variability between replicate cores in that particular treatment. Nonetheless, in systems such as Narragansett Bay that are undergoing mandated nutrient reductions, it is plausible that the balance of N fixation and denitrification could shift, increasing the contribution of N fixation to overall net  $\text{N}_2$  fluxes.

While the impact of N enrichment on salt marsh N fixation and denitrification has been examined in other studies, additional transplant studies such as this one would be extremely useful in understanding how increases and decreases in fertilization affect marshes with various histories of anthropogenic N loading. In particular, longer-term studies that track changes over time and using genomics to examine the impact to microbial communities could particularly enhance our understanding of these processes and their responses to anthropogenic N enrichment over seasonal, annual and multi-year time scales.

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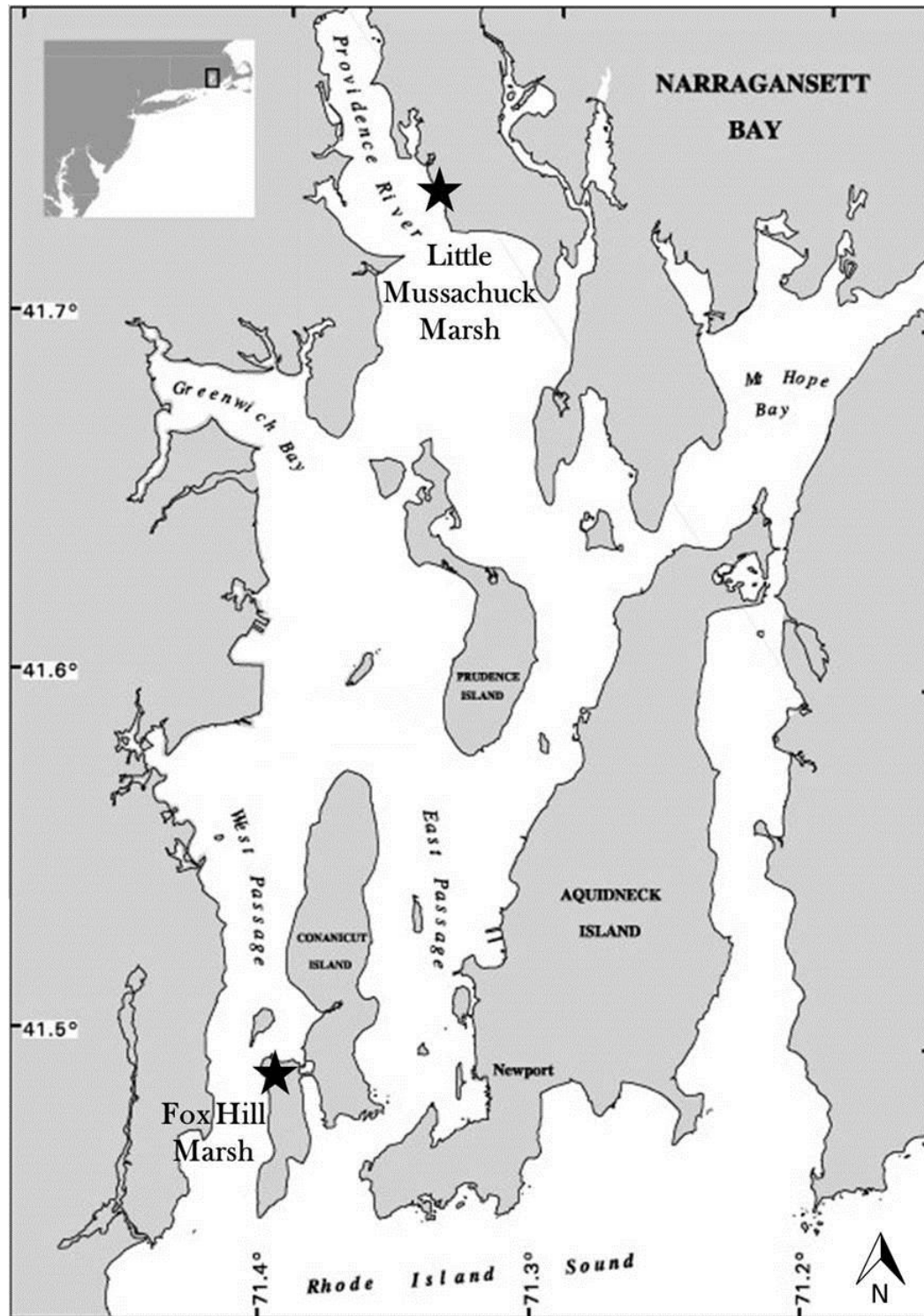


Figure 3-1. Map showing locations of study sites in Narragansett Bay, Rhode Island. Map of Narragansett Bay courtesy of <http://www.gso.uri.edu/phytoplankton/>. Data provided by RIGIS.

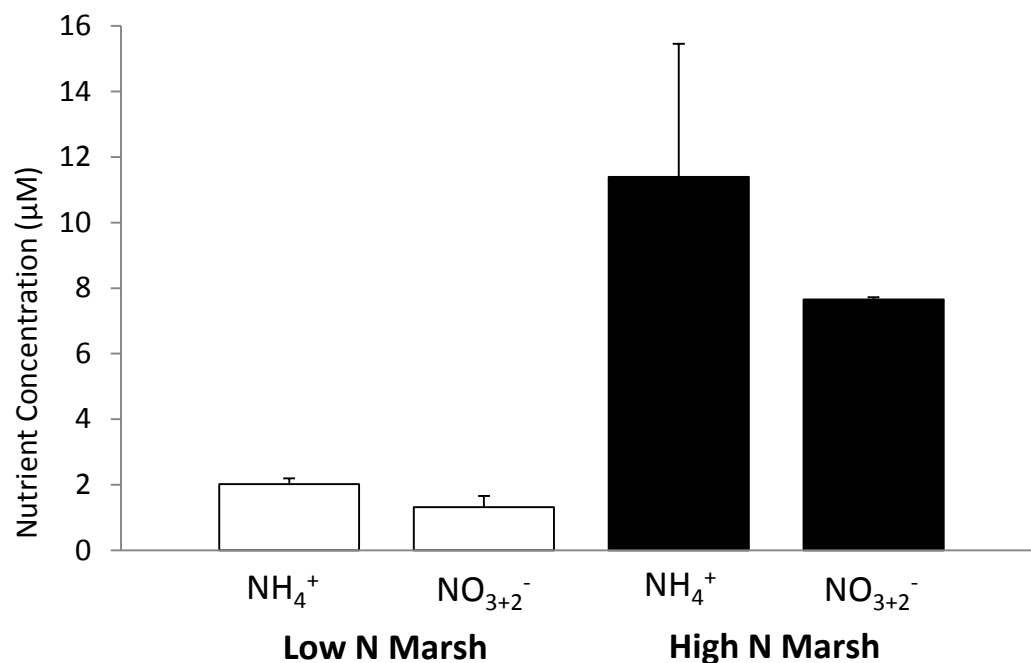


Figure 3-2. Ambient ammonium ( $\text{NH}_4^+$ ) and nitrate + nitrite ( $\text{NO}_{3+2}^-$ ) concentrations ( $\mu\text{M}$ ) in the surface tidal water. Concentrations are averaged from the two collection dates (10/6/11 and 10/12/11) for the nitrogen (N) fixation and denitrification incubations. The “Low N Marsh” refers to Fox Hill and the “High N Marsh” refers to Little Mussachuck. The error bars represent standard error of the averaged concentrations.

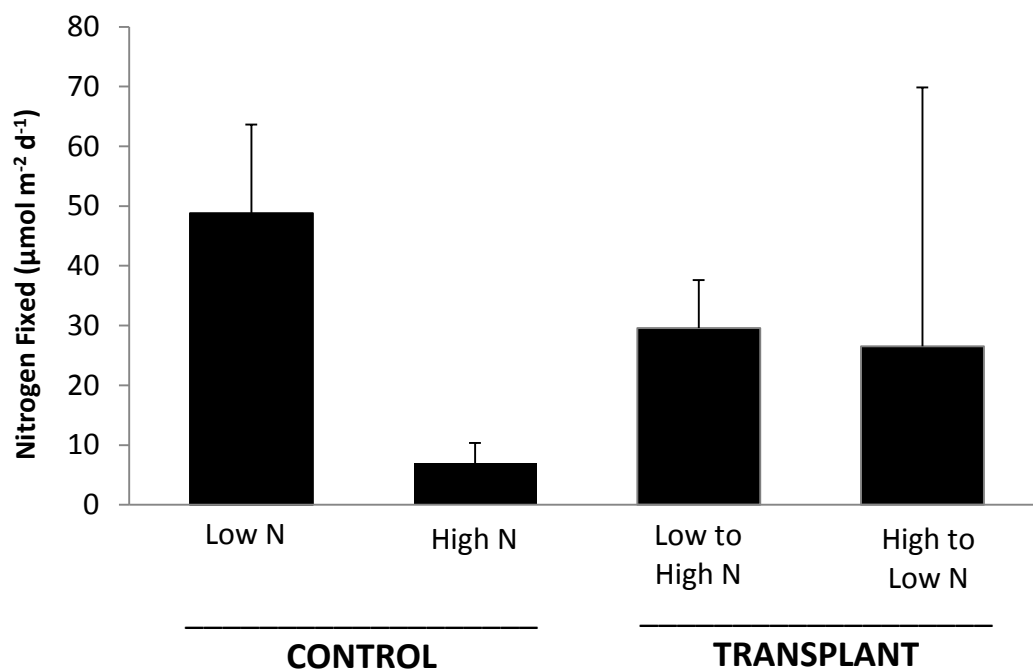


Figure 3-3. Nitrogen (N) fixation ( $\mu\text{mol m}^{-2} \text{d}^{-1}$ ) measured in the cores among the four treatments. “Control” refers to the treatment in which sediment cores were re-planted in their marsh of origin – either the “Low N” marsh (Fox Hill) or the “High N” marsh (Little Mussachuck). “Transplant” refers to cores re-planted in the opposite marsh. Error bars represent standard error of the slope from linear regressions.

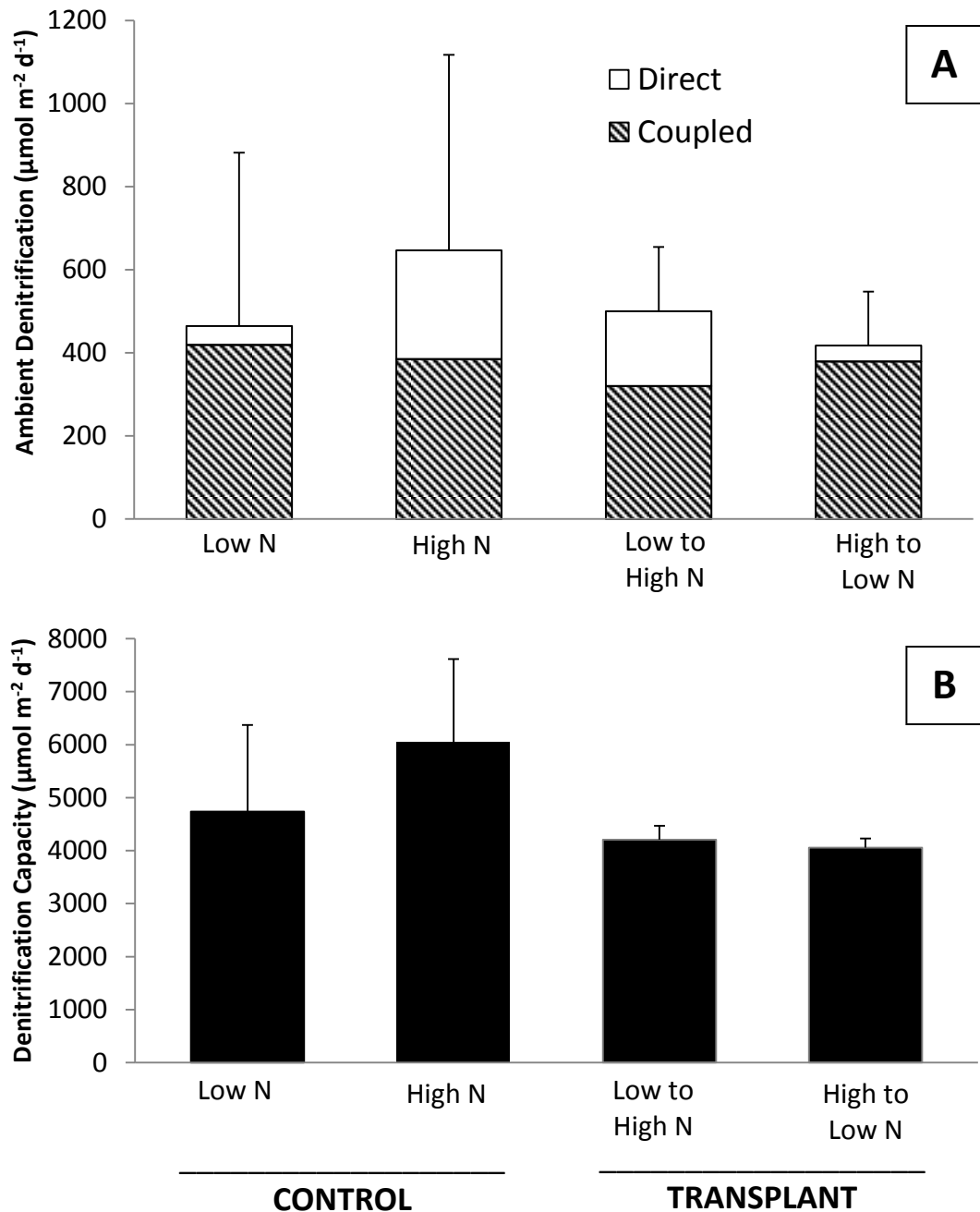


Figure 3-4. Denitrification activity ( $\mu\text{mol m}^{-2} \text{d}^{-1}$ ) measured in the cores among the four treatments. (A) Coupled nitrification-denitrification and direct denitrification, which in total represent ambient denitrification, and (B) denitrification capacity. “Control” refers to the treatment in which sediment cores were re-planted in their marsh of origin – either the “Low N” marsh (Fox Hill) or the “High N” marsh (Little Mussachuck). “Transplant” refers to cores re-planted in the opposite marsh. Error bars represent standard error of the slope from linear regressions for total ambient denitrification (A) and denitrification capacity (B).

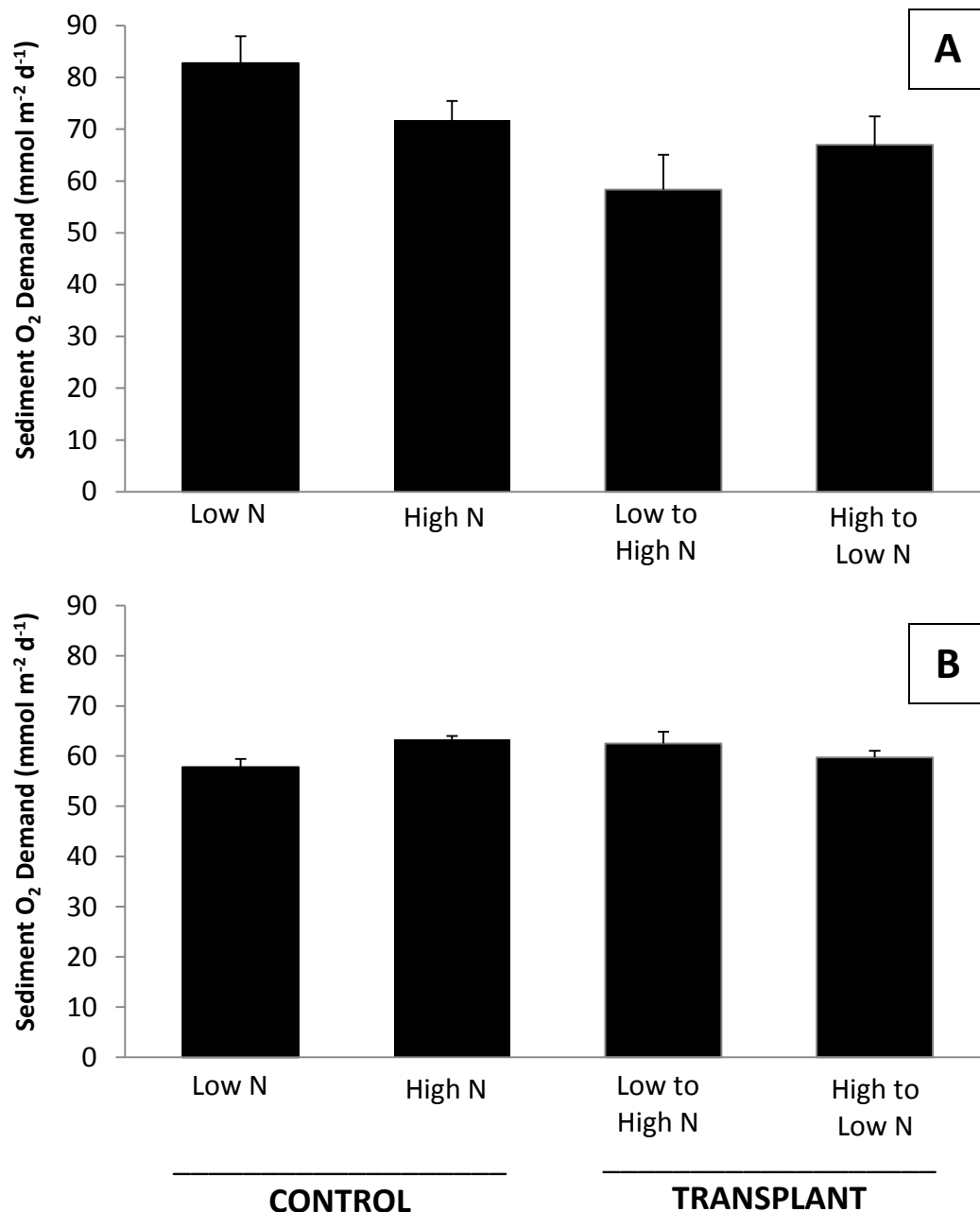


Figure 3-5. Sediment oxygen (O<sub>2</sub>) demand (SOD) measured in the cores among the four treatments. **(A)** SOD from the nitrogen (N) fixation incubation and **(B)** SOD from the denitrification incubation. “Control” refers to the treatment in which sediment cores were re-planted in their marsh of origin – either the “Low N” marsh (Fox Hill) or the “High N” marsh (Little Mussachuck). “Transplant” refers to cores re-planted in the opposite marsh. Error bars represent standard error of the slope from linear regressions.

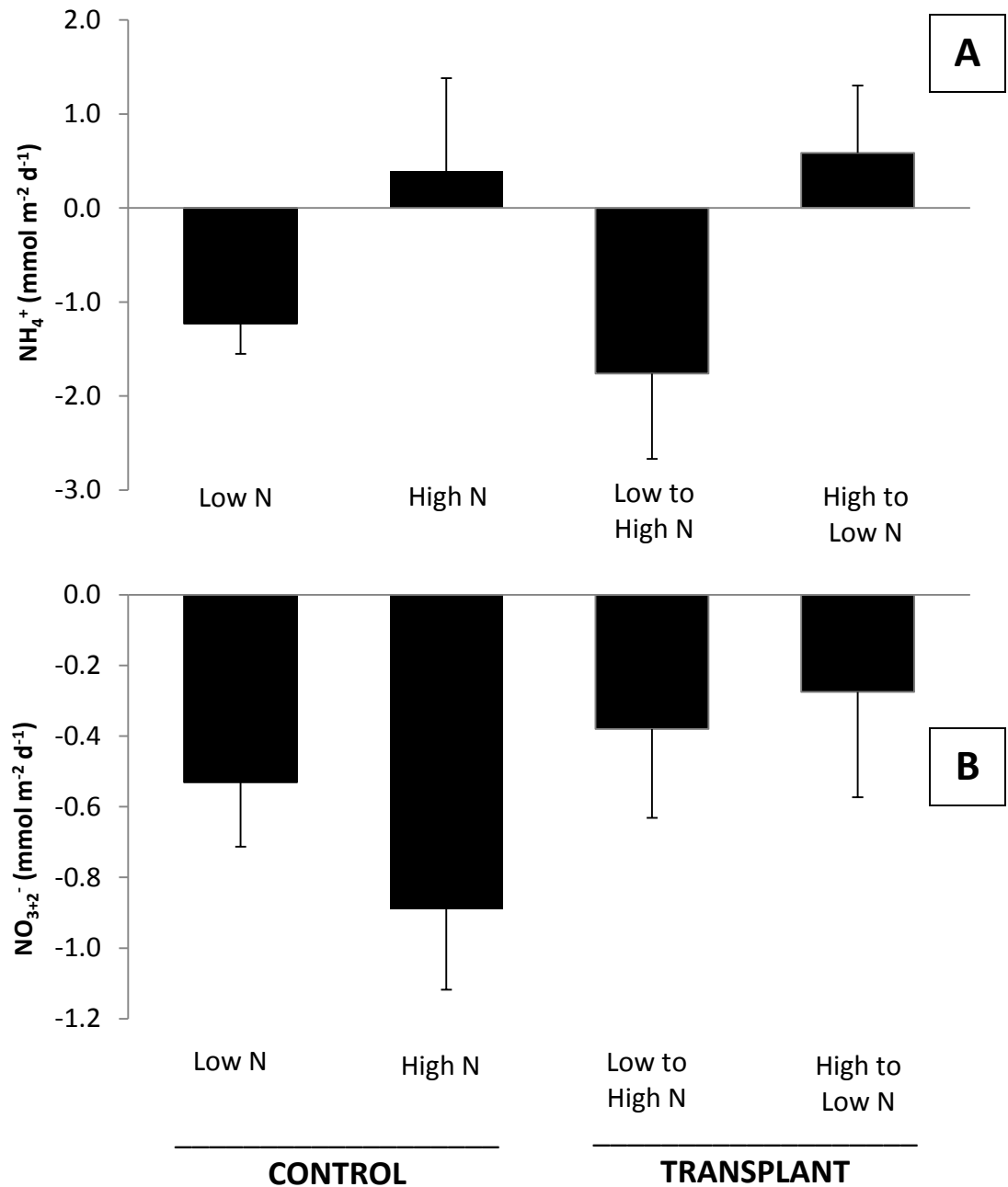


Figure 3-6. Nutrient fluxes measured in cores from the nitrogen (N) fixation incubation among the four treatments. **(A)** Ammonium ( $\text{NH}_4^+$ ) and **(B)** nitrate + nitrite ( $\text{NO}_{3+2}$ ) fluxes between the sediment-water interface. “Control” refers to the treatment in which sediment cores were re-planted in their marsh of origin – either the “Low N” marsh (Fox Hill) or the “High N” marsh (Little Mussachuck). “Transplant” refers to cores re-planted in the opposite marsh. Error bars represent standard error of the slope from linear regressions.

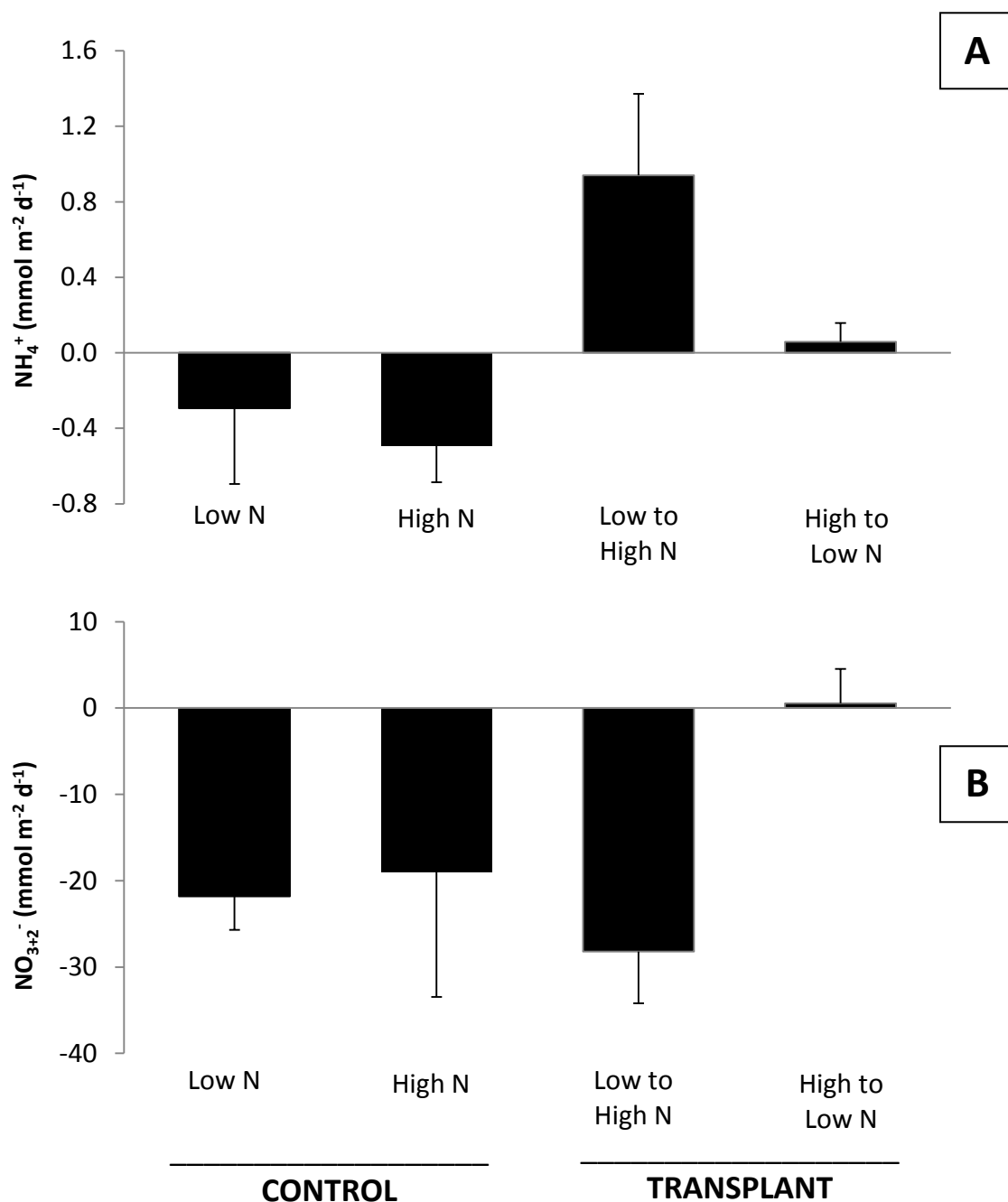


Figure 3-7. Nutrient fluxes measured in cores from the denitrification incubation among the four treatments. **(A)** Ammonium ( $\text{NH}_4^+$ ) and **(B)** nitrate + nitrite ( $\text{NO}_{3+2}^-$ ) fluxes between the sediment-water interface. “Control” refers to the treatment in which sediment cores were re-planted in their marsh of origin – either the “Low N” marsh (Fox Hill) or the “High N” marsh (Little Mussachuck). “Transplant” refers to cores re-planted in the opposite marsh. Error bars represent standard error of the slope from linear regressions.



## **APPENDIX A – ADDITIONAL METHODS AND CALCULATION DETAILS**

### **A-1. CALCULATIONS FOR PRODUCTION/UPTAKE OF N<sub>2</sub>, ETHYLENE, AND NUTRIENTS IN INTACT CORE INCUBATIONS: CORRECTING FOR DILUTION**

For the intact core incubation method to measure denitrification, N-fixation, and nutrient uptake and production, I present the following calculations used to correct and extrapolate all measured rates.

Using a gravity-fed flow-through sampling system to collect overlying water samples without introducing air bubbles or creating a vacuum, seawater stored in carboys flowed into the surface of the cores each time I collected a sample. Therefore, the overlying water (and constituents being measured) became diluted with carboy water during each sampling event.

#### **Overlying water samples**

- 1) At T<sub>0</sub> (first sampling event), I did not need to correct for dilution and calculated the total moles of N<sub>2</sub>/ethylene/NH<sub>4</sub><sup>+</sup>/NO<sub>3+2</sub><sup>-</sup> in the overlying water as follows:

$$Total\ mol = C_1 * V_{over}$$

Where

C<sub>1</sub> = concentration of N<sub>2</sub>/ethylene/NH<sub>4</sub><sup>+</sup>/NO<sub>3+2</sub><sup>-</sup> at T<sub>0</sub>

V<sub>over</sub> = volume of overlying water

- 2) The volume of the core is calculated as such:

$$V_{over} = h * \pi r^2$$

Where

h = 40 cm (height of water column)

r = 5 cm (radius of core)

therefore V<sub>over</sub> = 3.1416 L in all cores

- 3) At all subsequent sampling events of overlying water, I calculated the total moles of  $\text{N}_2/\text{ethylene}/\text{NH}_4^+/\text{NO}_{3+2}^-$  and corrected for dilution resulting from previous sampling using the following equations:

$$\text{Total mol at } T_1 = (C_1 * V_{\text{over}}) + (C_0 * V_{\text{removed}})$$

$$\text{Total mol at } T_2 = (C_2 * V_{\text{over}}) + (C_0 * V_{\text{removed}}) + (C_1 * V_{\text{removed}})$$

$$\text{Total mol at } T_3 = (C_3 * V_{\text{over}}) + (C_0 * V_{\text{removed}}) + (C_1 * V_{\text{removed}}) + (C_2 * V_{\text{removed}})$$

Where

$T_0$  = First sampling event

$T_1$  = Second sampling event

$T_2$  = Third sampling event

$T_3$  = Fourth sampling event

$C_0$  = concentration of  $\text{N}_2/\text{ethylene}/\text{NH}_4^+/\text{NO}_{3+2}^-$  at  $T_0$

$C_1$  = concentration of  $\text{N}_2/\text{ethylene}/\text{NH}_4^+/\text{NO}_{3+2}^-$  at  $T_1$

$C_2$  = concentration of  $\text{N}_2/\text{ethylene}/\text{NH}_4^+/\text{NO}_{3+2}^-$  at  $T_2$

$C_3$  = concentration of  $\text{N}_2/\text{ethylene}/\text{NH}_4^+/\text{NO}_{3+2}^-$  at  $T_3$

$V_{\text{over}}$  = total volume of overlying water

$V_{\text{removed}}$  = volume of water removed during sampling

The following volumes of samples were removed (and therefore diluted) during each sampling event ( $V_{\text{removed}}$ ):

- Denitrification incubations – 115 mL total (20 mL x 2 samples for  $\text{N}_2$  gas, 75 mL for nutrients)
- Nitrogen-fixation incubations – 175 mL total (50 mL x 2 samples for ethylene gas, 75 mL for nutrients)

### **Porewater + overlying water mixture samples**

I corrected for dilutions outlined above for any samples taken after  $T_0$ . I also had to make further calculations to account for additional sampling procedures. Prior to sampling porewater, I siphoned off the top half of the water column to reduce the amount of dilution of the porewater into the water column and to make it logistically easier to break up the sediment. I measured the volume of overlying water that I siphoned off to later account for the moles of  $\text{N}_2/\text{ethylene}/\text{NH}_4^+/\text{NO}_{3+2}^-$  in the siphoned water.

In the process of breaking up the sediment and mixing it into the overlying water, I added a bromide tracer to track the dilution of porewater into overlying water. I added

the tracer after siphoning (but before breaking up sediment) and collected two samples (7 mL each) for analysis of bromide concentration. I then broke up top 4-5 cm of sediment with a large metal fork, gently mixed the sediment + porewater + overlying water together, then sampled for N<sub>2</sub>/ethylene, NH<sub>4</sub><sup>+</sup> and NO<sub>3+2</sub><sup>-</sup>, and bromide.

Therefore for each core, to the total concentration of N<sub>2</sub>/ethylene/NH<sub>4</sub><sup>+</sup>/NO<sub>3+2</sub><sup>-</sup> in the porewater-overlying water mix, I had to account for the volume of the total mixture, in addition to correcting for dilution (as shown above).

- 1) For example, below is the calculation for a core that was sacrificed and sampled for porewater at T<sub>1</sub> (second sampling event):

$$\begin{aligned} \text{Total mol in porewater + overlying mix at } T_1 \\ = (C_{1 \text{ mix}} * V_{\text{mix}}) (C_{1 \text{ over}} * V_{\text{siphoned}}) + (C_{0 \text{ over}} * 0.175L) \\ + (C_{1 \text{ over}} * 0.175L) \end{aligned}$$

Where

$T_1$  = Time period 1 (second sampling event)

$C_{1 \text{ mix}}$  = concentration of N<sub>2</sub>/ethylene/NH<sub>4</sub><sup>+</sup>/NO<sub>3+2</sub><sup>-</sup> at T<sub>0</sub> in porewater + overlying water mixture

$C_{0 \text{ over}}$  = concentration of N<sub>2</sub>/ethylene/NH<sub>4</sub><sup>+</sup>/NO<sub>3+2</sub><sup>-</sup> at T<sub>0</sub> in overlying water

$C_{1 \text{ over}}$  = concentration of N<sub>2</sub>/ethylene/NH<sub>4</sub><sup>+</sup>/NO<sub>3+2</sub><sup>-</sup> at T<sub>1</sub> in overlying water

$V_{\text{siphoned}}$  = Volume of water siphoned out of core

$V_{\text{mix}}$  = Volume of porewater-overlying mix

- 2) The volume of the porewater-overlying water mix was determined using the following equation:

$$V_{\text{mix}} = \frac{Br_{\text{added}} (mg)}{[Br_{\text{total}}] \text{ in mix } \left( \frac{mg}{L} \right) - [Br_{\text{ambient}}] \left( \frac{mg}{L} \right)}$$

Where

$V_{\text{mix}}$  = Volume of porewater-overlying mix

$Br_{\text{added}}$  = Bromide added to overlying water (includes ambient bromide)

$Br_{\text{ambient}}$  = Ambient bromide in the seawater

$Br_{\text{total}}$  = Total added and ambient bromide in the porewater + overlying water mixture

## **A-2. CALCULATIONS FOR PRODUCTION/UPTAKE OF N<sub>2</sub>, ETHYLENE, AND NUTRIENTS IN INTACT CORE INCUBATIONS: CONVERSIONS, REGRESSIONS, AND EXTRAPOLATIONS**

### **Denitrification**

- 1) Dissolved <sup>29</sup>N<sub>2</sub>, and <sup>30</sup>N<sub>2</sub> gases analyzed by the membrane inlet mass spectrometer (MIMS) were converted from the instrument output to concentrations using the expected concentrations of <sup>28</sup>N<sub>2</sub> and <sup>29</sup>N<sub>2</sub> in standard water bath held at constant temperature and in equilibrium with the atmosphere.
- 2) Total  $\mu\text{mol}$  of <sup>29</sup>N<sub>2</sub>, and <sup>30</sup>N<sub>2</sub> produced in the cores were calculated using the equations and procedures outlined in A-1 and then averaged between the two replicate samples.
- 3) Total <sup>29</sup>N<sub>2</sub>, and <sup>30</sup>N<sub>2</sub> from the porewater + overlying water mix was plotted over time, and the slope of linear regressions were used to generate production ( $\mu\text{mol}$  of N<sub>2</sub>/hour). See Appendix A-3 for an example linear regression.
- 4) Denitrification rates were calculated using equations from the isotope pairing technique (Nielsen 1992). See Appendix A-5 for equations and descriptions of the method.
- 5) Hourly denitrification rates were converted to  $\text{mmol/day}$  and extrapolated to  $\text{m}^2$  by multiplying by a factor of 127.389 (because the surface area of the cores was  $78.54 \text{ m}^2$ ).

### **Nitrogen Fixation**

- 1) Peak areas from the gas chromatograph (GC) were converted to total  $\text{nmol}$  of ethylene (in a  $50 \text{ mL}$  sample) using a linear equation derived from a standard curve that was concurrently run on the GC.
- 2) The concentration of ethylene ( $\text{nmol/L}$ ) was calculated by dividing by  $50\text{mL}$  and multiplying by 1000 to convert from  $\text{mL}$  to  $\text{L}$ .
- 3) Total  $\text{nmol}$  of ethylene produced in the cores was calculated using the equations and procedures outlined in A-1 and then averaged between the two replicate samples.
- 4) Total ethylene in the porewater + overlying water mix was plotted over time, and the slope of linear regressions were used to generate production ( $\text{nmol}$  ethylene/hour). See Appendix A-3 for an example linear regression.
- 5) Ethylene production was converted into N-fixation rates using the stoichiometric ratio of 3:1 (ethylene rates were divided by 3).

- 6) Hourly N-fixation rates were converted to  $\mu\text{mol}/\text{day}$  and extrapolated to  $\text{m}^2$  by multiplying by a factor of 127.389 (because the surface area of the cores was  $78.54 \text{ m}^2$ ).

### Nutrients

- 1) Nutrient concentrations ( $\mu\text{M}$ ) from the Lachat autoanalyzer were converted to total  $\mu\text{mol}$  (produced or taken up) (including dilution corrections) using the equations and procedures outlined in A-1. However, it should be noted that the dilution calculations for nutrients accounted for the ambient nutrient concentrations present in the seawater prior to beginning the incubations. For example:

$$\text{Total mol at } T_1 = (C_1 * V_{\text{over}}) + ((C_0 * V_{\text{removed}}) - C_{\text{ambient}})$$

Where

$T_1$  = Second sampling event

$C_0$  = concentration of  $\text{NH}_4^+/\text{NO}_{3+2}^-$  at  $T_0$  (first sampling event)

$C_1$  = concentration of  $\text{NH}_4^+/\text{NO}_{3+2}^-$  at  $T_1$

$C_2$  = concentration of  $\text{NH}_4^+/\text{NO}_{3+2}^-$  at  $T_2$

$C_{\text{ambient}}$  = ambient concentration of  $\text{NH}_4^+/\text{NO}_{3+2}^-$

$V_{\text{over}}$  = total volume of overlying water

$V_{\text{removed}}$  = volume of water removed during sampling

- 6) Total  $\mu\text{mol}$  of nutrients in the porewater + overlying water mix was plotted over time, and the slope of linear regressions were used to generate production ( $\text{nmol } \text{NH}_4^+ \text{ or } \text{NO}_{3+2}^- \text{ per hour}$ ). See Appendix A-3 for an example linear regression.
- 7) Hourly production/uptake rates were converted to  $\text{mmol}/\text{day}$  and extrapolated to  $\text{m}^2$  by multiplying by a factor of 127.389 (because the surface area of the cores was  $78.54 \text{ m}^2$ ).

### A-3. CALCULATIONS FOR PRODUCTION OF N<sub>2</sub> IN INTACT CORE INCUBATIONS: EXAMPLE REGRESSIONS

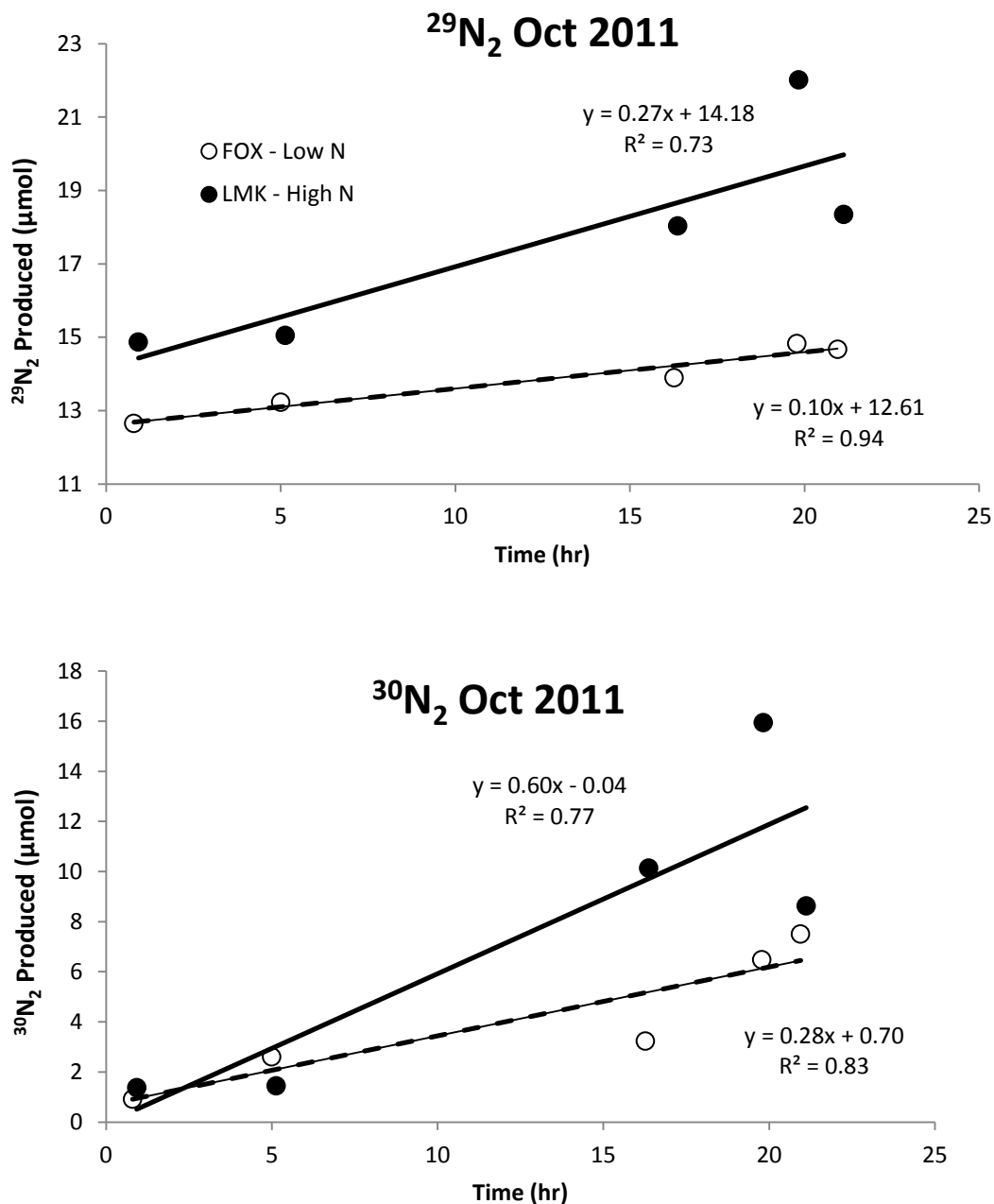


Fig. A-3. Production of <sup>29</sup>N<sub>2</sub> and <sup>30</sup>N<sub>2</sub> produced in the top 3-6 cm of sediment in cores collected from two marshes, Fox Hill (FOX) and Little Mussachuck (LMK). Each point represents measurements averaged from two replicate samples taken from one core. At every time point, one core from each site was sacrificed in order to collect porewater in the top layer of sediment.

**A-4. CALCULATIONS FOR PRODUCTION OF ETHYLENE IN INTACT  
CORE INCUBATIONS: EXAMPLE REGRESSIONS**

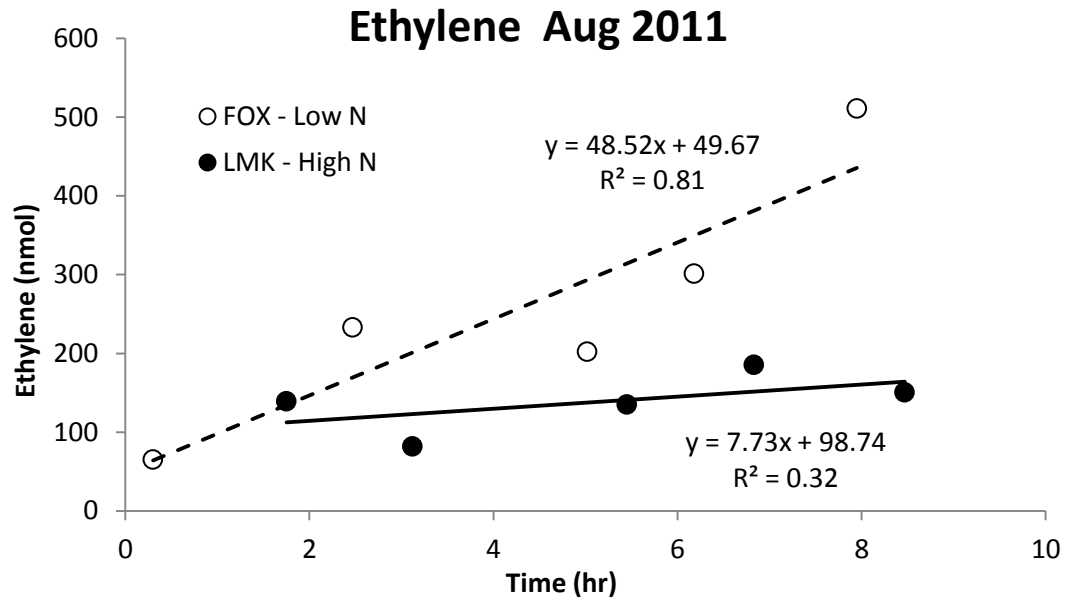


Fig. A-4. Production of ethylene produced in the top 3-6 cm of sediment in cores collected from two marshes, Fox Hill (FOX) and Little Mussachuck (LMK). Each point represents measurements averaged from two replicate samples taken from one core. At every time point, one core from each site was sacrificed in order to collect porewater in the top layer of sediment.

**A-5. CALCULATIONS FOR PRODUCTION OF ETHYLENE IN SEDIMENT  
PLUG INCUBATIONS:  
CONVERSIONS AND EXTRAPOLATIONS**

- 1) Peak areas from the gas chromatograph (GC) were converted to total nmol of ethylene (produced from a 5 mL sediment plug) using a linear equation derived from a standard curve that was concurrently run on the GC.
- 2) Blank samples were made by injecting acetylene gas into vials containing 5 mL of inert rubber (in place of sediment). Any ethylene present in the acetylene gas measured from the blanks was subtracted from all of the incubation samples.
- 3) Ethylene production was standardized per gram of dry sediment (all sediments used in the incubations were dried at 60°C for 4 hours), and divided by the incubation time, resulting in rates of nmol ethylene produced  $\text{g}^{-1} \text{h}^{-1}$ .
- 4) The rates were then standardized per  $\text{cm}^2$  area of a sediment core. This was done using bulk density measured on separate cores, which gave us site and depth-specific ratios of volume to grams dry weight.
- 5) Hourly production/uptake rates were converted to  $\mu\text{mol}/\text{day}$ , extrapolated to  $\text{m}^2$  by multiplying by a factor of 127.389 (because the surface area of the cores was  $78.54 \text{ m}^2$ ), and then averaged between the two replicate samples.
- 6) To scale the rates to a depth of 5cm in order to compare them to the whole core measurements (which were estimated to reach a depth of 3-6cm), we depth-integrated the rate for the 0-2cm section of sediment (multiply the rate by 2) and for the 2-5cm section of sediment (multiply the rate by 3) and added them together.



## A-6. CALCULATION OF STANDARD ERROR OF LINEAR REGRESSIONS

The equation used to calculate the standard error of the slope (i.e. rate) of the linear regressions:

$$S_{yx} = \sqrt{\frac{\sum(y - \bar{y})^2 - \frac{[\sum(x - \bar{x})(y - \bar{y})]^2}{\sum(x - \bar{x})^2}}{n - 2}}$$

Where:

y = actual y variable

$\bar{y}$  = predicted y variable (based on the regression equation)

x = actual x variable

$\bar{x}$  = predicted x variable (based on the regression equation)

n = number of x,y points in the regression

Source:

<http://www.okstate.edu/ag/agedcm4h/academic/aged5980a/5980/newpage24.htm>

## A-7. BROMIDE TRACER TESTS TO MEASURE DEPTH PENETRATION OF AMENDED SEAWATER DURING PRE-INCUBATION OF INTACT SEDIMENT CORES

Table A-7. Results from amended-seawater saturation tests using “pre-incubation” methods employed in our whole core incubations. Average bromide (Br) concentrations and the percent of bromide-amended seawater in the porewater of sediments are shown, including standard error. The tests were run with replication of  $n = 4$  cores. The seawater had an original Br concentration of  $580 \mu\text{M}$  and the amendment increased the Br concentration to  $3009 \mu\text{M}$ .

	Ave [Br] $\mu\text{M}$	<i>s.e.</i> [Br] $\mu\text{M}$	Ave % Amended SW	<i>s.e.</i> % <i>Amended</i> <i>SW</i>
2-HOUR INCUBATION				
<u>Little Mussachuck</u>				
0 - 2 cm	2027	254	58.8	9.8
2 - 4 cm	1702	369	46.2	14.3
4 - 6 cm	1320	273	31.5	10.6
6 - 8 cm	1138	247	24.4	9.6
<u>Fox Hill</u>				
0 - 2 cm	1584	278	41.7	10.8
2 - 4 cm	1453	282	36.6	10.9
4 - 6 cm	1455	297	36.7	11.5
6 - 8 cm	1590	247	41.9	9.6
4-HOUR INCUBATION				
<u>Little Mussachuck</u>				
0 - 2 cm	2860	176	91.1	6.8
2 - 4 cm	2430	243	74.4	9.4
4 - 6 cm	2379	278	72.5	10.8
6 - 8 cm	2123	427	62.5	16.5
<u>Fox Hill</u>				
0 - 2 cm	1609	331	42.6	12.8
2 - 4 cm	1436	401	36.0	15.5
4 - 6 cm	1108	366	23.3	14.2
6 - 8 cm	1038	323	20.6	12.5

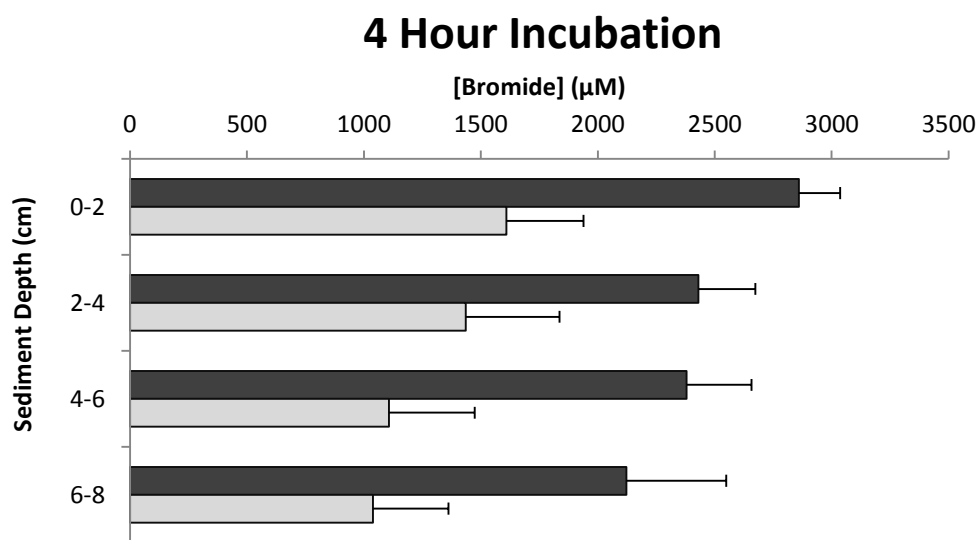
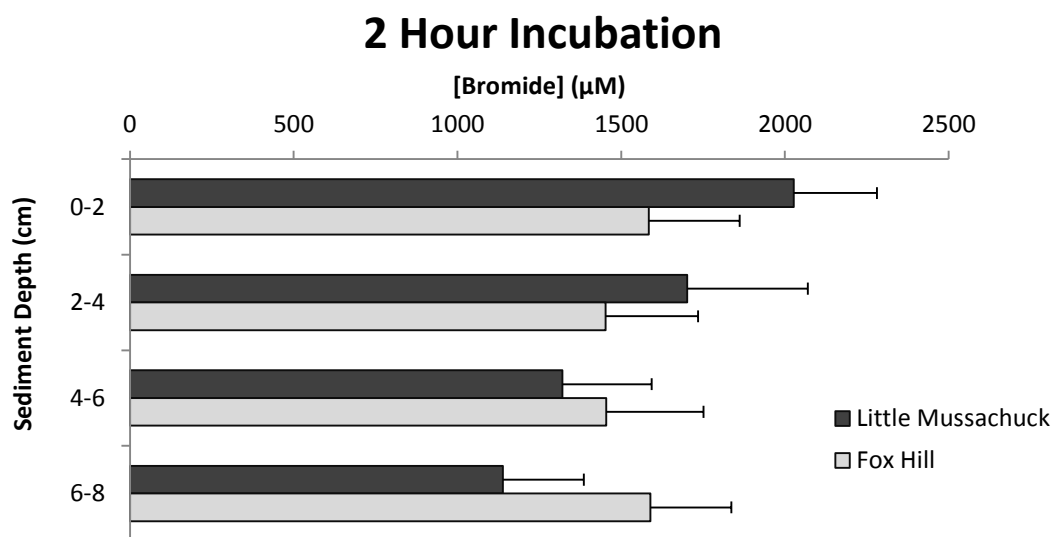


Figure A-7. Bromide (Br) concentrations ( $\mu\text{M}$ ) in porewater extracted from sediment horizons of intact cores incubated for 2 or 4 hours with Br-amended seawater. The seawater had an original Br concentration of  $580 \mu\text{M}$  and the amendment increased the Br concentration to  $3009 \mu\text{M}$ . The amended seawater then was added to the cores and allowed to slowly drain through the bottom of the cores (using the same methods of the “pre-incubations” from the whole core incubations to measure denitrification and nitrogen fixation). Error bars represent standard error of four replicate cores from each marsh.

# **A-8. COMPARISON OF OVERLYING ONLY VERSUS POREWATER + OVERLYING MIX RATES FOR AMBIENT DENITRIFICATION**

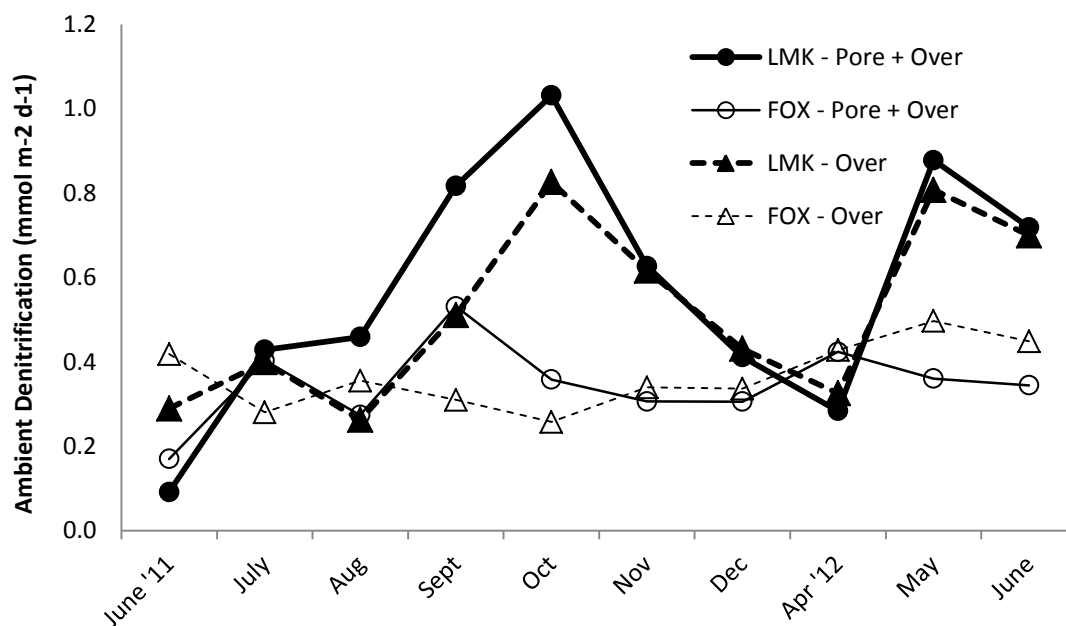


Figure A-9. Rates of ambient denitrification (D14) calculated from two different sampling methods: 1) using samples of overlying water only (“Over”) or 2) using samples of porewater + overlying water slurries produced by breaking up the top 4cm of sediment and mixing it into the overlying water (“Pore + Over”). Rates for the two marshes are shown: Little Mussachuck (“LMK”) and Fox Hill (“FOX”).

# **A-9. AMBIENT TIDAL NUTRIENTS FROM DENITRIFICATION AND NITROGEN FIXATION INCUBATIONS**

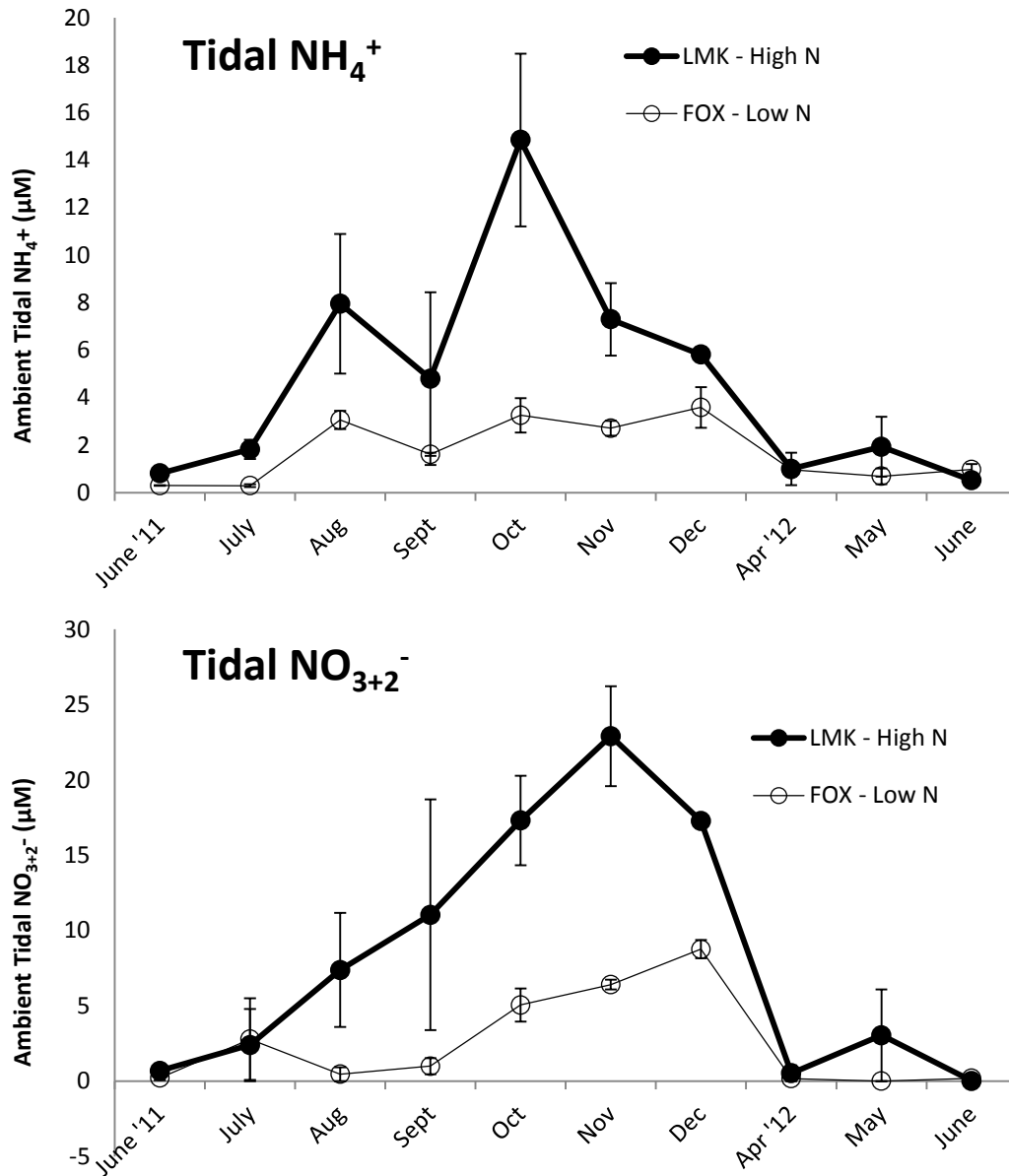


Figure A-10. Monthly ambient tidal ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate + nitrite (NO<sub>3+2</sub><sup>-</sup>) concentrations averaged between the denitrification and nitrogen fixation incubations for the two marsh study sites, Little Mussachuck (LMK) and Fox Hill (FOX). Error bars represent standard error of the averaged values.

# **A-10. SEDIMENT OXYGEN DEMAND FROM DENITRIFICATION AND NITROGEN FIXATION INCUBATIONS**

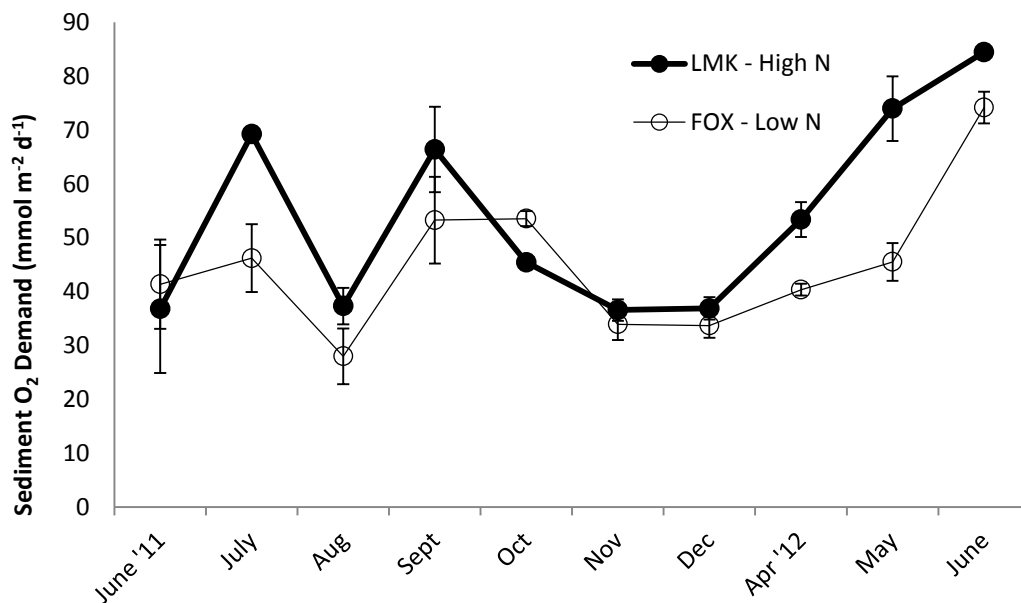


Figure A-11. Monthly sediment oxygen (O<sub>2</sub>) demand measured in the sediment cores averaged between the denitrification and nitrogen fixation incubations for the two marsh study sites, Little Mussachuck (LMK) and Fox Hill (FOX). Error bars represent standard error of the averaged values.

## A-11. TIDAL INUNDATION AND FLOODING DATA

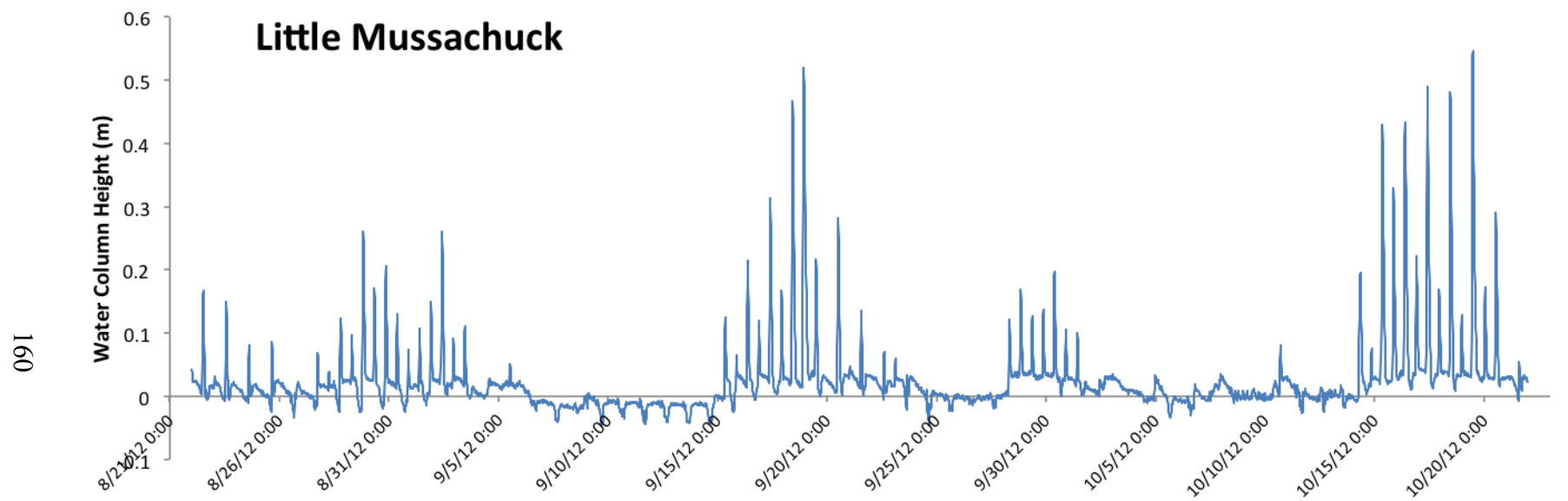


Figure A-11. (A) Tidal flooding data at Little Mussachuck marsh from 8/21/11 to 10/22/11. Inundation and depth of the water column were measured at 15-minute intervals using a HOBO U20 Water Level Logger on the sediment surface.

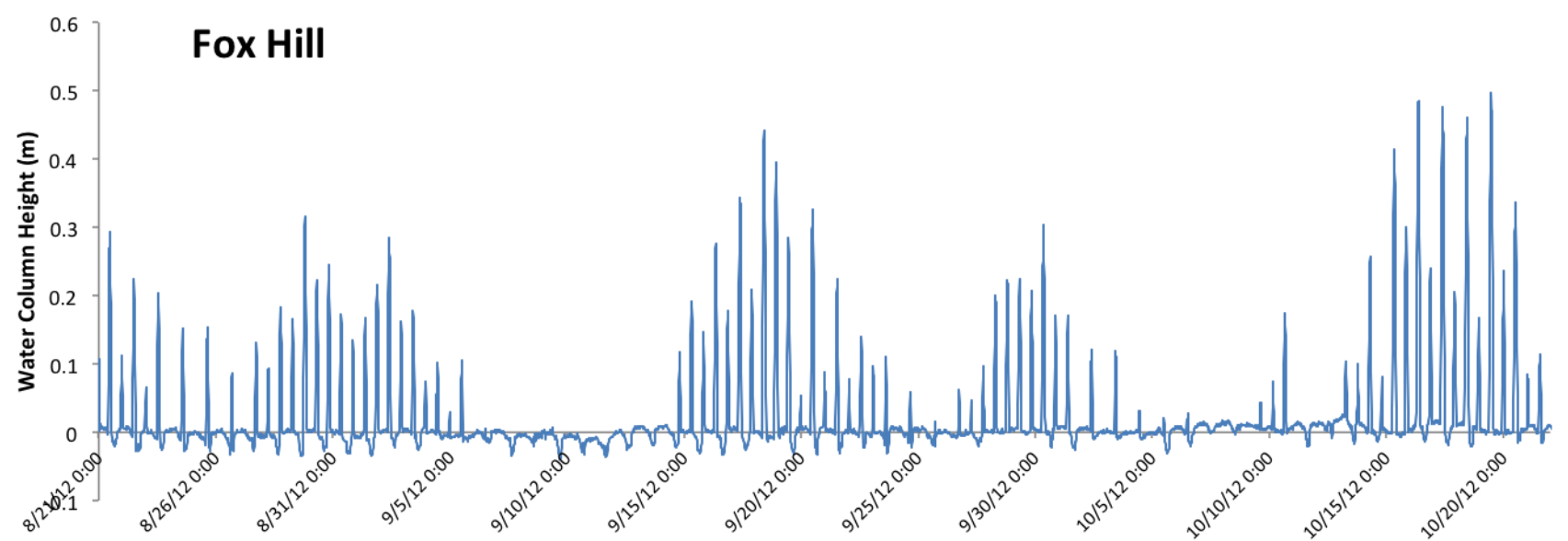


Figure A-11. (B) Tidal flooding data at Fox Hill marsh from 8/21/11 to 10/22/11. Inundation and depth of the water column were measured at 15-minute intervals using a HOBO U20 Water Level Logger on the sediment surface.



## **A-12. TRANSPLANT EXPERIMENTAL DESIGN AND METHODS CONSIDERATIONS**

### ***Comparison of Transplant Data to Ambient Data***

To gain some insight into unintended effects of the transplantation process, we compared N fixation, denitrification, and SOD results from our “Control” cores (Ch. 3) to ambient measurements made in October 2011 for our seasonal studies (Ch. 1 and Ch. 2). In the ARA incubations N fixation from this experiment was 53% lower at Fox Hill and 87% lower at Little Mussachuck compared to ambient rates measured three weeks earlier in September. SOD rates from this study were 35% higher at Fox Hill and 4% lower at Little Mussachuck. The incubation temperatures were the same between N fixation studies, and so we attribute differences in activity to possible disturbance from the transplantation process that depressed N fixation, or the potential leaching of C from freshly cut roots in the ambient September incubation that boosted N fixation.

In the IPT incubations, we compared ambient coupled nitrification-denitrification and denitrification capacity, as these rates would likely be unaffected by differences in tidal water  $\text{NO}_3^-$  between studies. In the cores from this study, coupled denitrification was 37% higher at Fox Hill and 10% lower at Little Mussachuck than ambient rates measured five days later in October. Denitrification capacity was higher in the cores from this study, 10% higher at Little Mussachuck and 100% higher at Fox Hill. In fact, the denitrification capacity measured in the Control cores at Fox Hill from this study was much higher than any other measurements of

denitrification capacity that we made over an annual cycle. SOD rates were 17% and 48% higher in this study. The higher rates of denitrification and SOD in the IPT incubation from this study could be due to, in part, an incubation temperature that was 2.5°C higher than the temperature from the ambient October measurements.

### ***Experimental Design Considerations***

Although the transplant experiment was largely a success in examining the impact of N regimes on marsh sediment N cycling, there are a number of factors in the experimental design that need to be considered. First, the act of cutting through roots and sediment to extract the cores and replant them was an obvious source of disturbance. Over the three month period following re-planting however, roots grew in to fill the space between the extracted core and “hole” into which they were planted. In many cores, small shoots began to grow out of the surface sediments, showing indications of recovery and acclimation of the plants and belowground biomass in the cores. The mesh that surrounded the re-planted cores could have had additional effects. However we believe these would have been minimal, as the wide mesh side and small amount protruding from the surface border of the cores likely had a minimal effect on slowing water flow or shading the cores. We also noticed that roots grew through the mesh below the sediment surface, and we did not see any excess growth of algae within the cores or on the mesh.

The Control treatments, in addition to supplying direct comparisons of N fixation and denitrification between the two marshes, provided a means to quantify unintended effects of the transplantation process on N fixation and denitrification

activity. We were able to compare activity in our Control cores to other incubations that we conducted in two separate studies (Ch. 1 and Ch. 2). In these additional studies, we collected and immediately incubated cores from Little Mussachuck and Fox Hill on a monthly basis over the course of one year, using the exact same incubation methods as the ones employed in this study. In both cases, the cutting of belowground biomass to extract the cores could have disturbed the microbial communities and very likely caused some leaching of dissolved organic carbon (DOC) into the sediments from broken roots and rhizomes.

A major factor that is important to consider in how the experimental design influenced the outcome of the study is the timing. First, the length of time that the cores remained in the marshes following transplantation is important. As evidenced by the results outlined in Chapter 3, the Transplant cores seemed to be influenced by a legacy effect of being exposed long-term to a particular N regime. A longer period of time for the transplantation, perhaps an annual cycle, may give the microbial communities and their associated biogeochemical activity time to acclimate to the new environment. The dynamics of the plants and belowground biomass and physiology, which can be affected by N regime (e.g. Wigand et al. 2003) may also be impacted by the length of time of the transplant.

Another aspect of timing to consider is the seasonal timing of when the cores were transplanted and when they were collected and incubated. Tidal  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations are low in the summer at both sites (Figure A-11). Because our cores were transplanted in July, they likely did not experience a large difference in tidal N regime until early fall when DIN concentrations increased at Little Mussachuck. This

could have delayed a response to N enrichment in the cores transplanted into Little Mussachuck. In addition, our monthly data (Ch. 1 and Ch. 2) demonstrate that denitrification and N fixation rates tended to differ most dramatically in the fall. If we had performed the transplant experiment earlier in the year and measured denitrification and N fixation activity in the summer (when ambient rates between marshes were similar), we may not have seen any differences among treatments in the experiment.

Spatial constraints also influenced and limited our study. We specifically focused on examining the effects of tidal N regime on N fixation and denitrification activity within surface vegetated sediments. Though we did not capture activity deeper within the sediments and our rates are likely underestimates, we assumed that the influence of tidal DIN on microbial N cycling was likely limited to the surface sediments. In a marsh fertilization study, Koop-Jakobsen and Giblin (2010) found that direct denitrification (which reduces external  $\text{NO}_3^-$  from tidal waters) was minimal in sediments below 5cm. In addition, other sources of external N, such as groundwater and surface runoff, may impact marsh N cycling, but we assumed that tidal N is the most important source of N inputs to the surface sediments of the low marsh. Groundwater inputs likely flow much deeper in the marsh and are intercepted by the creek banks and creek bottoms (Howes et al. 1996). The marsh border and high marsh likely intercept surface runoff, though runoff may contribute to N inputs in the low marsh during precipitation events.

Finally, we acknowledge that a major limitation in our experimental design is the lack of statistical replicates. The whole core incubation set-up we used, with 12

cores total per incubation, was very time-consuming and logistically complicated to execute. Therefore, to increase replication in future work it would be prudent to also conduct separate incubations, such as sediment plugs, that more feasibly allow for higher replication. Fortunately, despite the lack of statistical replication in our study we observed differences among treatments in denitrification and N fixation activity. However, increased replication would undoubtedly help to clarify some results, such as the high variability we found in our N fixation “High to Low N Transplant” treatment.

#### **LITERATURE CITED**

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